Development of vegetables with improved consumer quality: A case study in Brussels sprouts.

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Dit proefschrift wordt opgedragen aan mijn vader († 10 juli 1976), mijn eerste supporter in de wetenschap.

Stellingen

1. Het glucosinolaatgehalte van spruitkoolrassen is afhankelijk van rassenkeuze en teeltomstandigheden en wordt in hoge mate bepaald door architectuurkenmerken van het wortelstelsel en de beschikbaarheid en verdeling van sulfaat in de grond.
Dit proefschrift.

2. De sensorische waarneming van smaakbepalende kenmerken is nationaliteits gebonden: Niet-bittere spruitjes zijn "zoet" in Engeland en "gewoon lekker" in Nederland.
Dit proefschrift.

3. De verervingsresultaten van het sinigrine en progoitrine gehalte in spruitkool tonen aan dat de selectie op multigene eigenschappen efficient kan verlopen.
Dit proefschrift.

4. De beschermende rol van plantaardige secundaire inhoudsstoffen zal nimmer afdoende kunnen worden vastgesteld bij de huidige gebrekkige (bio)chemische karakterisering van voedingsmiddelen.
Clapper et al. Preclinical and clinical evaluation of broccoli supplements as inducers of glutathione S-transferase activity. Clinical Cancer Res. 3 (1997), 25-30.

5. Auteurs die onderzoeksgegevens fragmenteren over meerdere korte publicaties, om veelvuldig wetenschappelijk te kunnen scoren, zouden moeten weten dat veel niet synoniem is met goed.
Nijhoff et al. Effects of consumption of Brussels sprouts on intestinal and lymphocytic glutathione S-transferase in humans. Carcinog. 16 (1995), 2125-2128.
Nijhoff et al. Effects of consumption of Brussels sprouts on plasma and urinary glutathione S-transferase class-α and -$\square$ in humans. Carcinog. 16 (1995), 955-957.

6. De aanhoudende stroom van opmerkingen over de mogelijke toxiciteit van glucosinolaten voor mens en dier is gebaseerd op anekdotische informatie.

7. De introductie van lange houdbaarheid in groentes zal leiden tot een geografische verschuiving van teeltgebieden, niet tot een verhoogde totale afzet of betere prijs.

8. De stelling: "Over smaak valt niet te twisten" dient gewijzigd te worden in "Smaak leidt tot twisten".

9. Als muzikanten hun inspiratiebronnen zouden moeten vermelden op dezelfde wijze als auteurs van wetenschappelijke publicaties, zou de tekst van sommige liedjes voor meer dan de helft uit referenties bestaan.

10. De vraag naar met vitamine C verrijkte chips in winkels schreeuwt om aardappels met een hoger vitamine C gehalte.

11. De kunst van spruitkoolveredeling inspireert de spruitkoolkunst.
(Cover en discussie van) dit proefschrift.

Stellingen bij het proefschrift 'Development of vegetables with improved consumer quality: A case study in Brussels sprouts', te verdedigen door Hans van Doorn op 29 september 1999.

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Chapter 1
General Introduction

Economical and nutritional value of vegetables.

Vegetables comprise a major part of the world's annual food production and are produced in various forms under a wide range of climatological conditions. In 1997, 558.6 million tons of vegetables were produced globally with a value of 43.7 billion DFL. The Netherlands is responsible for 13% of the value of the global vegetables production (Annual Report Dutch Productschap Tuinbouw, 1997). Vegetables have a low caloric value and a high nutritional value and form a considerable part of the daily human intake of minerals such as calcium and iron, fibres, vitamin C, carotenoids and folate. Vegetables contribute for about 11% to the daily energy intake of UK consumers (Annual Report Ministry Agriculture, Fisheries and Food, UK, 1997). Worldwide, a considerable part of the population is involved with the production, transport and marketing of vegetables. In the Netherlands more than 17.000 growers and approximately 7000 companies are involved with, respectively, the production and marketing of vegetables (Annual Report Dutch Productschap Tuinbouw, 1997). The vegetable business contributes significantly to the gross national product of most countries in the world. Vegetables have therefore an important nutritional and economical value.

Recent evolution of vegetable production: Altered organisation and quality criteria.

The present assortment of vegetables has been selected and optimized for human consumption for many centuries. Until relatively recently, specific crops were only produced at sites where all their essential growing conditions were met naturally. The geographical distribution of crops is also significantly determined by the appreciation of ethnic groups for specific vegetables; e.g. dry beans are typically grown in hot relatively dry locations with a predominantly Hispanic background. Hot peppers mainly are appreciated by Asian and Mexican consumers. A rather slow, low capacity transport network and distribution system

ensured that typically foreign vegetables were until recently only available in limited quantities in export markets.

Recent technological developments, e.g. irrigation systems, improved glasshouse design, education and development aid have made it possible to grow many crops at locations which were previously unsuitable for this purpose. A highly developed infrastructure facilitates the transport and distribution of formerly exotic crops all over the world. These developments in conjunction with socio-economic developments have turned the cultivation, distribution and marketing of vegetables into a rapidly changing and highly specialized business. The worldwide cultivation of specific crops has led to an increase in production levels, a saturation of the market and in specific periods even to an overproduction of specific crops.

Although production of many crops is still concentrated regionally, others such as tomato, pepper, melon, cauliflower,
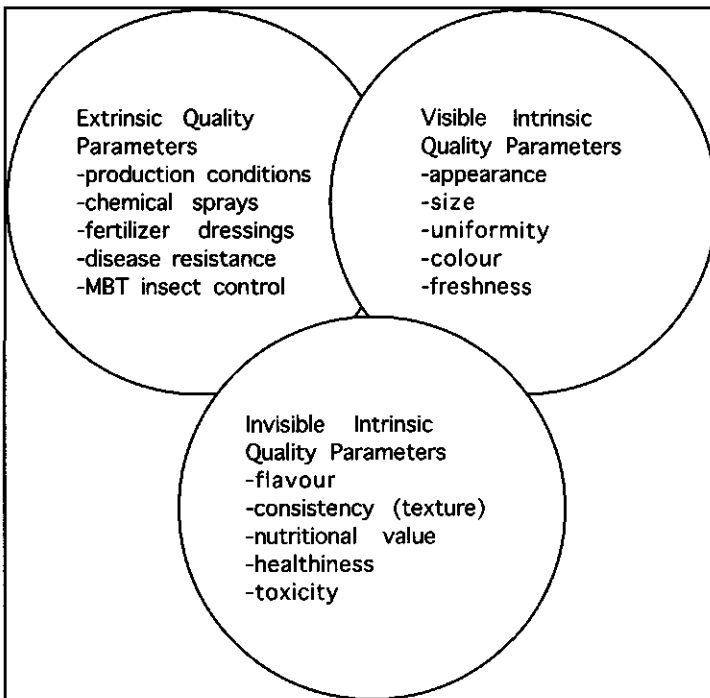

Figure 1. Clusters of relevant quality parameters of vegetables for the vegetable business chain (VBC).

broccoli and lettuce are produced almost worldwide and exported to the markets of interest. Crops which are globally distributed have to meet a more diverse set of quality criteria compared to locally produced and traded crops, as the former crops need to be transported over long distances without loss of quality. Product quality criteria can be subdivided into extrinsic quality parameters, which describe how vegetables are cultivated, and intrinsic quality aspects, e.g. visible traits such as the appearance, uniformity, freshness and colour of vegetables. The main quality criteria for vegetables are presented in figure 1.

Some intrinsic aspects have a non-visible character and include features such as flavour, consistency and nutritional value. Currently, the extrinsic and visible intrinsic quality parameters are most important for almost all members of the vegetable business chain (VBC). The extrinsic and (in)visible intrinsic quality parameters interact with each other to a certain degree as presented in figure 1.

Consumer preferences for vegetables.

The large scale production of vegetables with an optimal visible quality, but lacking appropriate flavour profiling, seems to be the main reason why the demand for tasty vegetables by consumers is growing steadily. Modern consumers not only ask for vegetables with an excellent appearance but have a pronounced interest in vegetables that combine factors such as flavour, consistency, storability, digestibility and an appropriate content of health promoting compounds. Consumers have consistent meanings with respect to flavour and health related compounds in vegetables. Some vegetables, such as Brussels sprouts, are disliked because of a too strong flavour (Fenwick et al., 1983; Bedford, 1989). Other crops such as tomato are not appreciated because of a lack of flavour (Kopeliovitch et al., 1982). Cabbage can be disliked because of the production of off-flavour (Maruyama, 1970).

The demand for vegetables with improved intrinsic quality is further strengthened by other factors. Firstly, supermarkets offer a variety of fruits and vegetables which has led to consumers becoming more critical in their choice. Secondly, the intrinsic

quality of specific vegetables is highly variable depending on cultivar choice, cultural methods and country of production. The consequence is that consumers cannot buy vegetables with a constant quality that meet their expectations. Thirdly, specific groups of consumers have a healthy life style and follow diets which are significantly comprised of vegetables with a high extrinsic and intrinsic quality. In the last few years consumers are evolving from passive into assertive members of the VBC and contribute to the current quality profile of vegetables in terms of extrinsic and intrinsic quality parameters.

Business chain reversal and the branding of vegetables with improved taste.

The demand of consumers for vegetables with high intrinsic quality has created a new hierarchy in the VBC. The VBC is partly reversing from a top-driven - into a bottom-driven structure. Consumers now determine the composition and quality of the vegetable assortment in shops and supermarkets for a significant part. The vegetable business chain (VBC) has many members and applies alternative approaches to serve the various types of consumers. Currently, four different marketing types can be recognized for vegetables: (1) the consumer driven channel, (2) the marketing driven channel, (3) the high productivity channel and (4) the old road. Figure 2 gives an overview of the VBC in terms of members and the four different approaches to supplying the consumer with vegetables. The top panel shows the partners in the vegetable business chain from seeds distributors on the left, to retailers on the right. The lower panel shows the 4 channels which are currently used for the marketing of vegetables. The four channels demand vegetables with specific characteristics in terms of quality and quantity. On the one hand, the consumer - and marketing driven channels are both customer-driven and use dedicated (contract) growers for their vegetable production. The consumer driven channel comprises innovative top retailers, who obtain their top quality produce from market oriented suppliers, and create added value by processing the
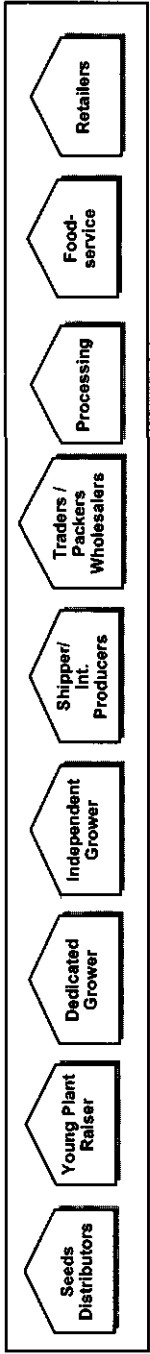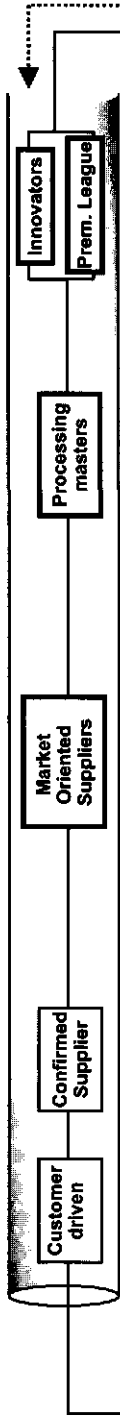
Figure 2. The design of the vegetable business chain in terms of members (upper panel) and approaches to supply vegetables to consumers (lower panel).

vegetables. The marketing driven channel comprises marketing oriented retailers who are supplied with high quality produce by pro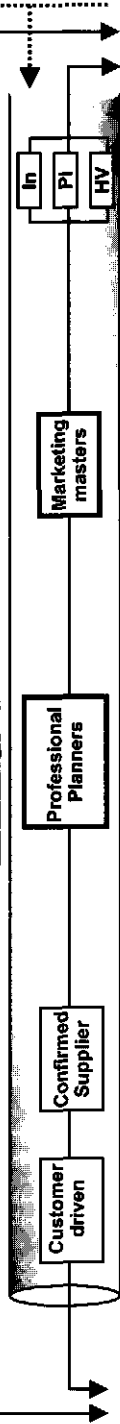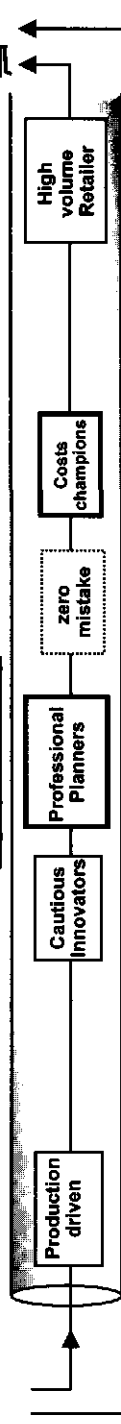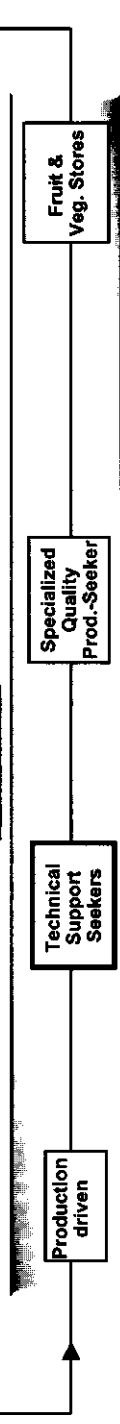fessional planners. The vegetable throughput through the former channels, in terms of quality and composition, is based on interactions between consumers and the business partners in the channels. On the other hand the high productivity channel and the old road are both production driven and depend on independent growers for their, on many occasions, standard quality vegetables. The high productivity channel has high volume retailers who depend for most of their sales on a relatively low price. The classical fruit and vegetables stores are found in the old road. They are supplied with produce by specialized traders and wholesalers.

All four VBC channels consider visible quality factors such as appearance, uniformity, freshness and colour of vegetables to be important parameters for their customers but give different priorities to extrinsic quality parameters and invisible intrinsic quality traits. The consumer driven channel gives high priority to environmentally friendly vegetable production and uses intrinsic quality aspects such as flavour, consistency and nutritional value to attract and satisfy their very demanding consumers. In contrast, the high production channel is almost not interested in extrinsic and invisible intrinsic quality parameters and requests a high yield of standard intrinsic quality produce.

The consumer driven channel of the VBC is a clear example of the influence of consumers on the marketing of vegetables. The new trend in consumer behaviour has initiated a discussion in the VBC and breeding companies about the status of quality criteria. Despite their initial reluctance, many members of the VBC are quickly anticipating further developments and have started to introduce new vegetable brands with specifications for extrinsic and intrinsic quality traits such as supersweet Brussels sprouts and top flavour tomatoes. This anticipation is mainly stimulated by potentially higher profits due to a shift of sales from the low margin classical vegetables which are produced in high volumes to high-quality high margin brand vegetables. E.g. Dutch auctions have, in collaboration with grower associations, started to segment tomatoes for flavour after the drop in sales of Dutch

tomatoes in the main German export market. Within two years, tomatoes were marketed in various brands with special attention to colour and flavour (e.g. Bon Appetit), way of picking (e.g. truss tomatoes), shelf life (e.g. Reif und Fest) and culture conditions (e.g. the butterfly logo for the control of insects via MBT). These brands however are often still comprised of tomatoes from various cultivars and originate from different growers and therefore do not strictly have a homogeneous quality in time.

At present the production of vegetables with excellent quality characteristics is still mainly a matter of cultivation methods and opportunistic marketing. Improvement of aspects such as flavour in vegetables by cultivar choice is practiced on a small scale, mainly because reliable information regarding the flavour of cultivars is scarcely available for most crops.

The percentage of consumers interested in invisible intrinsic quality aspects is still growing thus creating opportunities for the development of tasty, healthy and nutritious vegetables. Some seed companies and specific members of the VBC are ready to supply consumers with vegetables of high intrinsic and extrinsic quality which originate from high quality cultivars. The development of such cultivars and control of their quality is still a matter of time and effort.

Desirable and conflicting quality criteria.

Breeding companies play a key role in the development of new cultivars which are intended to meet the demands of all members of the VBC. It is almost impossible to combine all possible quality criteria into one cultivar: a cultivar is always a compromise. Breeding companies have, due to pressure from the VBC, optimized most crops for intrinsic quality aspects such as uniformity and appearance. In most crops, resistances to the major pests and diseases have been introduced with a significant positive influence on these traits.

Currently, the VBC almost completely determines which extrinsic and intrinsic quality criteria new cultivars of crops have to meet. For some aspects, all members of the chain share the same opinion. Other aspects are more contradictory between

channels of the VBC. Table 1 gives an overview of the quality perception of the different members of the business chain.

An aspect such as environmentally friendly production is of general interest for most members of the VBC. Resistant cultivars are convenient for the growers, do not have to be sprayed with relatively expensive fungicides or pesticides, do not contain residues, are desired by most consumers and can be promoted as healthy by all members of the VBC.

Table 1. Quality criteria for members of the VBC.

VBC member	desirable quality issues
breeder	new cultivars with better performance than the standard in the market with respect to the key quality traits
grower	productivity, uniformity, disease resistance
auction	uniformity, reliable supply, constant quality
distribution	shipability, keepability, availability, damage sensitivity
retailer	good shelflife, diversity, appearance, low waste
consumer	tasty, healthy, sustainable, convenient, constant quality

A good flavour, although desired by many consumers, is generally an aspect with low priority for the VBC. Independent growers in the high production channel are hardly interested in producing vegetables with an improved flavour when they are paid equally for less tasteful cultivars that have a higher yield and better performance. Many exporters and distributors dislike segmentation for flavour in vegetables because diversification in general restricts their flexibility. The consumer driven channel with innovative top retailers, promote vegetable flavour to increase their profit (capture extra added value)

Another contradictory trait in the business chain is long shelf life (LSL), a trait which has been successfully introduced in crops like tomato, melon, pepper, iceberg lettuce and cucumber. For melon and tomato, classical cultivars of these crops with a conventional short shelf life have almost been wiped out of the

export market by the cultivars with LSL. The tomato cultivar Daniela and the melon cultivar Clipper have proved that LSL has a high economical value for growers and all other members of the business chain, but this LSL is accompanied by less desirable traits for the consumers.

LSL is for crops such as tomatoes and melons an ideal trait to increase their storability on the one hand, but significantly decreases their flavour on the other hand. Especially in fruit vegetables, the development of aromas is directly linked to the ripening process. Long shelf life tomatoes, for example, never turn completely ripe and, as a consequence, lack the aroma production of full red tomatoes. The aroma of tomatoes is determined by ketones and aldehydes which are derived from fatty acids and carotenoids (Buttery et al., 1987, 1988 and 1989). LSL tomatoes have a relatively low carotenoid content and, consequently, little aroma (Kopeliovitch et al., 1979 and 1982). LSL Charentais melon cultivars have a reasonable taste because of their high sugar content but lack the production of the pleasant aroma which is characteristic for non-LSL Charentais cultivars.

Other contradictory quality parameters are used by members of the business chain and consumers. Intrinsic quality parameters such as uniformity, freshness, nice appearance are often obtained when vegetables are cultivated using an (over)optimal fertilizer level, optimal temperature and light intensity and sufficient irrigation. Optimization of these parameters results in high vigour and yield. Such conditions favour visible intrinsic parameters but often have a negative influence on taste. A good example of the negative influence of growth conditions on flavour is the relatively poor flavour of specific Dutch greenhouse tomato cultivars which have been over optimized for yield and intrinsic quality with poor flavour as a result. From the literature (e.g. Berry et al., 1988) we know that the yield of tomato cultivars is negatively correlated with the content of the flavour determining compounds such as sugar and organic acids in the fruits. Complaints from the German export market have renewed the emphasis on breeding for a good flavour in Dutch tomatoes.

In the current situation the large scale vegetable production with high yielding LSL cultivars has in general resulted in

products with a good intrinsic quality in terms of visible parameters, but often a poor flavour, aroma and consistency.

Breeding for improved intrinsic quality: The state of the art.

1. Classical breeding and (bio)technology.
Improvement of the intrinsic quality of specific crops can be achieved by classical breeding techniques which, when desirable, can be supported with the aid of (bio)technology. In many cases sufficient variation in the expression of flavour determining traits is available in the germplasm of crops to allow classic breeding. In some cases this variation is lacking and creation of variation via alternative techniques is necessary. Molecular engineering of crops is an option when adding or deleting specific traits. At present both classical breeding and molecular techniques are applied for the improvement of the intrinsic quality of vegetables (Gray et al., 1994). E.g. the flavour of tomatoes depends for a significant part on the rate of softening which is catalyzed by cell wall degrading enzymes such as pectinases (Carrington et al., 1993; Cheng and Huber, 1997). In tomato germplasm specific lines have been characterized having a very low rate of softening (Kopeliovitch et al., 1979). These lines are currently used for the breeding of firm tomato cultivars with long shelf life. In the ideal situation the suppression of pectinases in the tomato by molecular techniques also yields firm long shelf life tomatoes. Tomato cultivars with long shelf life which are based on both principles are now available on the market.

2. Improvement of quality with an integrated approach.
The development of vegetable cultivars with a high intrinsic quality seems for many reasons to be a job for the (global) breeding companies. Breeding companies have in general a broad range of genetic variation which is available for all relevant traits of crops and have the facilities to test the extent of the genetic and environmental contributions to the expression of specific traits. The breeding of cultivars which combine good intrinsic quality aspects such as flavour, shelf life and

consistency with intrinsic quality aspects is, however, only in an initial phase of development. Improvement of the intrinsic quality of vegetables needs an integrated approach as new cultivars need to combine quality traits with optimal field performance to satisfy the demands of the VBC.

3. Flavour of vegetables is determined by genetics and environment.

The low investment by breeding companies in improved intrinsic quality has a number of reasons. An enormous quantity of literature suggests that breeding for items such as improved flavour is not likely to be easy. Intrinsic quality attributes which are relevant for consumers, such as sweetness, bitterness, mealiness, acidity, consistency, are known to vary considerably in cultivars of the same crop, but are also significantly affected by the cultivation method (Petersen et al., 1998). Tomato cultivars, e.g., vary much with respect to sugar and acid content: the main taste determining factors (Hermann, 1979). The content of these compounds can also be manipulated considerably in a single cultivar by EC values used in the cultivation and irradiance profiles (Grierson and Kader, 1986). Interactions between cultivars and environmental parameters have a significant effect on the expression of intrinsic quality traits such as sugar and acid content. The fact that environmental parameters can determine up to 60% of the observed phenotypic variation of quality traits might explain why breeding for improved intrinsic quality is a time consuming process (Berry et al., 1988; Gull et al., 1989).

4. Many flavour determining compounds are characterized, and many are unknown.

In a number of cases the compounds responsible for typical flavour attributes have been identified and characterized. Good examples are sugars and organic acids as principles for sweetness and acidity in tomato (Jones and Scott, 1983; Stevens et al., 1979), sesquiterpene lactones as bitter principles in chicory (Leclercq, 1992), organic volatiles such as aldehydes and ketones as flavour principles in tomato (Buttery et al., 1987, 1988 and 1989), S-alkyl-cysteine-sulfoxides as pungency and flavour principles in onion and garlic (Fenwick and Hanley, 1985)

and glucosinolates as bitterness principles in *Brassica* vegetables (Fenwick et al.,1983). For many crops, however, the information concerning which compounds exert an influence on flavour is simply lacking or unclear.

Flavour research in fruit and vegetables, as conducted by institutes and universities, in general focuses on the identification and characterization of new compounds in single cultivars rather than on the determination of compounds that have a significant contribution to consumer acceptance (Hamatidou et al., 1992; Horvat and Senter, 1987; Wyllie and Leach, 1990).

Studies with respect to the content and significance of flavour determining compounds are often conducted on a small scale with much emphasis on the techniques that determine the identity and content of these specific compounds rather than on the variation of specific compounds in many cultivars of a crop (Springett et al., 1988). At present a vast number of highly sophisticated analytical tools are available to the modern scientist to isolate, characterize and quantify flavour and health promoting compounds in vegetables.

5. Limitations of breeding for improved quality.

Only part of the research results pertaining to flavour are useful in the improvement of the intrinsic quality of vegetables by breeding institutes and companies. The low application rate of methods for the determination of compounds with a positive effect on the intrinsic quality of vegetables in breeding programs can be explained by various reasons. The consumer appreciation of specific crops is often correlated to a complex array of flavour determining compounds which makes their application in large breeding programs impossible. For breeders, quality determining factors have to explain a significant part of the variation of a trait to be successfully applied.

It is essential that rapid, inexpensive assay methods are developed to replace the time consuming expensive analytical methods now used for the determination of flavour and health promoting compounds. Breeders work with extensive breeding programs and need methods that can be applied on a large scale to enable the fixation of traits in segregating inbred populations.

The inheritance of most flavour determining compounds has hardly been studied or only on a very limited scale. The content of flavour determining compounds does not inherit per se in a (simple) Mendelian fashion or with a low heritability. Even in ideal cases, the expression of a trait will be dependent on both the genetic background of cultivars and environmental conditions. The influence of both genetics and environment on a trait, and the heritability of the trait, are sometimes such that hardly any progress can be made in breeding for improved intrinsic quality. Breeding for increased sugar content in tomato has proven to be very slow due to a large environmental influence (Winsor and Adams, 1976).

Some compounds/traits have a pronounced negative influence on the field performance of crops and selection for the content of specific compounds can result in undesirable side-effects. Breeders only introduce new traits in crops when no negative influence is observed on all other relevant agronomic traits. E.g. breeding for tomatoes with a high sugar content is only possible when the yield is maintained at a high level. Physiological processes often have a negative impact on the biosynthesis and accumulation of flavour determining compounds.

For many intrinsic quality aspects, such as flavour and consistency, there is no universal agreement between consumer populations as to what is desirable. Consumers sometimes differentiate vegetables from specific crops into segments using their own characteristics. A consumer population can clearly be divided into subgroups with a preference for sour, sweet or aromatic tomatoes (Hobson and Bedford, 1989). A segmentation for pungent and non-pungent radish is observed in German consumers. Crops are only segmented for quality aspects when the segments have an economically significant volume in terms of quantity and value.

These examples clearly show that the scientific basis for supporting the breeding for improved invisible intrinsic quality in vegetables is small for breeding companies. Companies which nevertheless have initiated breeding activities for intrinsic quality traits in vegetables are at present still in an early phase of a difficult, expensive and time consuming process. Breeding

companies therefore consider carefully which intrinsic quality traits have to be introduced in breeding programs. Whether a new cultivar for a specific crop with an improved intrinsic quality will be a success depends, beside these technical aspects, also for a significant part on marketing and economical aspects.

Perspectives for vegetables with improved intrinsic quality.

1. Vegetables need to have a reliable quality.
Vegetables with improved intrinsic quality have an additional commercial value in the market compared to the standard bulk products. Whether improved products really become a success depends on factors such as consumer demand for the product, year-round availability, recognition, attractiveness and most of all the reliability of the product. The "FlavrSavr" tomato, historically the first biotechnologically engineered vegetable on the American market, is a good example of product branding for a claimed improvement in flavour and shelf life. The introduction of "FlavrSavr" failed because of inadequate logistics in the business chain and, despite the promises, an intrinsic quality not significantly better than standard tomatoes. In Europe the recent introduction of canned tomato paste prepared from an identically engineered processing tomato cultivar seems to be a success (Picton et al., 1995).

2. Vegetables need to have a minimum quality.
Breeding for a minimum quality in vegetables is another approach. Most vegetables vary considerably with respect to parameters which have an impact on flavour, consistency and shelf life. Consumer appreciation is consequently regularly negatively affected by the lack of flavour, the presence of off-flavours, too strong flavours or aberrant consistency.

Chicory (witloof), for example, is appreciated by consumers because of the presence of sesquiterpene lactones, compounds with a typical bitter taste (Leclercq, 1992). Too much lactucin, lactucopicrin and corresponding glucosides, however, are not appreciated by consumers and a significant percentage reject bitter witloof (own observations). It is advisable to breed for

witloof with an upper limit for bitterness to avoid complaints from the market about this aspect.

The same principle applies to tomato. Tomatoes for export to Germany should have a minimum flavour. Candidate new introductions from breeding companies are tested for flavour during official assessment trials. Vegetables must have a minimum (intrinsic) quality to prevent consumers from turning away from inferior products. The current assortment of cultivars of various crops present on the market has however hardly been evaluated for these aspects.

<u>Vegetables need to be healthy.</u>

Vegetables in general are not only eaten because of their attractive flavour and high nutritional value but also for their health promoting effects. Vegetables are an important source of dietary fibres, vitamins and secondary metabolites that prevent consumers getting cancer, high blood pressure, arterioscleroses etc. (Jakobey et al., 1988; Jansen et al., 1995 and references cited in it).

Secondary metabolites, such as glucosinolates and their degradation products in *Brassica* vegetables, induce the xenobiotic metabolism of consumers and are believed to prevent them getting colon cancer (Bradfield & Bjeldanes, 1987; Byers and Graham, 1984; Graham, 1984; Johnson et al., 1994; McDanell et al., 1988; Verhagen et al., 1993; Zhang et al., 1992). Compounds such as flavonoids (Wall et al., 1988a) and coumarins (Wall et al., 1988b) show antimutagenic activity in mutagenicity tests with the Salmonella T-98 strain and are believed to do so in humans. Vegetables with a high content of carotenoids, flavonoids, coumarins, glucosinolates and other bioactive secondary metabolites are highly ranked in the list of health promoting foods (Jansen et al., 1995). Compounds which increase the nutritional value, promote the health of consumers or improve the digestibility enhance the intrinsic quality of vegetables. However clear studies that unequivocally state that specific components benefit the human health are lacking. The role of glucosinolates and corresponding degradation products in health promotion is studied quite extensively. The health promoting activity of glucosinolates is demonstrated with epidemiology (Jansen et al.,

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1995) while the kinetics of phase I and II carcinogen conjugation and detoxification enzymes by glucosinolate derivatives is well defined in animal - and human intervention studies (Bradfield and Bjeldanes, 1987; Williamson et al., 1998; Bogaards et al., 1994; Nijhof et al., 1995a and b; Zhang et al., 1993). Cruciferous vegetables such as Brassicas contain a diversity of health promoting glucosinolates for consumers (Fenwick et al., 1983, Faulkner et al., 1998). The prolonged duration of human intervention studies as well as the diversity in consumption patterns of humans makes it, however, in general difficult to correlate specific internal compounds of leafy and fruity vegetables to health.

Some channels of the VBC are, nevertheless, already marketing specific vegetables or derived products which should prevent consumers from getting cancer, high blood pressure or other diseases which might be controlled by a vegetable diet (Clapper et al., 1997). Broccoli sprouts are currently available in US supermarkets as an alternative for cancer prevention. At present the VBC promotes vegetables as healthy in a general sense with no specifications with respect to the content of nutrients and health promoting compounds in specific vegetables most of the time. Packed fresh, frozen and canned vegetables are usually labeled with general information regarding the nutritional value in terms of kJoules, and the content of the main nutrients.

Vegetables which are grown in an organic way are the best practical example of a small, but essential, market segment with the predicate "healthy". Organic vegetables, however, derive the term healthy from the cultivation method, for instance without fertilizer, and not because of an enhanced level of health promoting compounds.

4. The balance between visible and invisible intrinsic quality parameters.

Much attention is given to the parameters that determine the intrinsic quality of vegetables offered to consumers: freshness, uniformity, maturity, ripeness, lack of damage. Items such as flavour and health are only scarcely mentioned in a crop such as tomato. The promotion of flavour and health related aspects might give vegetables a healthy status comparable as apples and

tropical fruits such as oranges and kiwis. The healthy status of apples and tropical fruits is not only the result of high content of health promoting compounds but also of long term promotion activities.

The recently implemented segmentation for taste and shelf life in tomato shows that diversification for quality attributes in vegetables has a future. In other crops, important quality parameters which attract consumers can also be identified.

Flavour and health related traits always have to be added to cultivars of specific crops which already have been optimized for all agronomic important traits such as yield, uniformity etc. Only then, will cultivars with an improved flavour and health image be successful in the market.

Breeding for improved quality traits: This thesis.

In this thesis an integrated approach is presented for the improvement of taste and shelf life of Brussels sprouts with classical breeding and the support of technology. Brussels sprouts is an attractive crop with respect to the expression of intrinsic quality parameters for most members of the VBC. Their typical flavour is dividing the consumer population into likers and dislikers. Brussels sprouts lack shelf life and have a healthy status based on a high content of vitamin C and health promoting glucosinolates. The improvement of taste is expected to make them more attractive for consumers and might lead to the consumption and production of a larger volume of Brussels sprouts. Better shelf life will prevent the post-harvest deterioration of sprouts in the VBC. Sprouts which maintain a fresh appearance after harvesting will give a low waste for retailers and remain attractive for consumers.

The poor taste of Brussels sprouts has for years been a major concern of the many members of the VBC and consumers. In *Brassica* vegetables, such as Brussels sprouts, a group of compounds named glucosinolates and their degradation products play a prominent role in plant defense against herbivores such as pigeons and deer (Boag et al., 1990; Giamoustaris and Mithen,

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1995), slugs (Giamoustaris and Mithen, 1995; Glen et al., 1990), non-host fungi (Drobnica et al., 1967; Esaki and Onozaki, 1982; Hejtmankova et al., 1979), host-specific fungi (Doughty et al., 1991; Greenhalgh and Mitchell, 1976; Mithen et al., 1986; Searle et al., 1982), nematodes (Lazzeri et al., 1993) and insects (Erickson and Feeny, 1974). Glucosinolates play a significant role in the attraction of host-specific insects. Glucosinolates also seem to be regarded negatively by consumers (Fenwick et al., 1983). This thesis describes in Chapter 2 and 3 the development of novel large scale assays for the determination of glucosinolates as alternatives for classic, but time-consuming, analytical methods such as gas chromatography (Heaney and Fenwick, 1980; Slominski and Campbell, 1987) and high performance liquid chromatography (e.g. Lewis and Fenwick, 1988). Our efforts have resulted in an alternative extraction method for glucosinolates and improved ELISA assays in comparison to those of Hassan et al. (1988). These assays have been applied in Chapter 4 to study the role of glucosinolates in the acceptance of Brussels sprouts by consumers. Consumer preference mapping resulted in the establishment of the critical level of glucosinolates for consumers and opened the way for the application of these assays for the definition of the heritability of the flavour-affecting glucosinolates in Brussels sprouts in Chapter 5. The characterization of environmental and phenotypic parameters which influence the content of glucosinolates in cultivars is described in Chapter 6. Chapters 2 to 6 essentially provide all the tools and information for the production of tasty Brussels sprouts for consumers and members of the VBC.

In chapter 7, a Brussels sprout breeding line will be presented with extreme long shelf life (LSL). LSL of fruits and vegetables is a complex trait which is under strict hormonal control. The hormones auxin, cytokinin and gibberellins are all found to contribute to the expression of the LSL trait in this sprout line. The trait is expected to be of high economic value to the VBC. Supermarkets and consumers will soon be able to store sprouts for a prolonged time under unfavourable conditions without a decrease in intrinsic product quality.

In the introduction to this thesis the importance of quality traits of vegetable crops for the business chain, consumers and breeding companies has been presented. The question whether breeding for improved quality traits in specific crops will find a permanent place in commercial breeding programmes has to be answered in the next few years.

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Chapter 2
Quantitative determination of the glucosinolates sinigrin and progoitrin by specific antibody ELISA assays in Brussels sprouts.

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Abstract

Glucosinolates from Brussels sprout samples were extracted using an effective concentration of 2% phosphoric acid followed by a neutralization step and heat treatment for removal of inactivated protein. The (potentially) bitter glucosinolates sinigrin and progoitrin were found to be stable during this new extraction protocol. Antisera, as raised against hemisuccinate-linked glucosinolate conjugates, were very specific in sandwich ELISA assays for their corresponding substrates. The ELISA assays showed maximally 7.4% cross-reactivity to other aliphatic glucosinolates and were log-linear from the nM to μM range. In comparison to the standard HPLC method, the sinigrin and progoitrin ELISA respectively slightly and considerably overestimate the actual content of these glucosinolates. The progoitrin content of samples as determined either with the ELISA assay or by HPLC, however, is highly correlated ($r^2=0.92$, n=12, p<0.01) suggesting that the former assay is also applicable for the screening of the progoitrin content in Brussels sprout samples.

Introduction

The taste and consumption quality of edible parts and seeds from cruciferous plants, such as Brussels sprouts, are significantly determined by glucosinolates, a group of thioglucosides with health-promoting or toxic properties for animals and humans. More than 90 glucosinolates have already been identified, which can be categorized in 3 groups consisting of aliphatic, aromatic and indole glucosinolates (Fenwick et al., 1983) depending on the chemical structure of their functional side chain. In crucifers, glucosinolates coexist with the enzyme thioglucosidase (E.C. 3.2.3.1), an enzyme with specific catabolic activity toward glucosinolates, which is located in isolated cells of (un)differentiated tissues (Thangstad *et al.*, 1990) strictly separated from its substrates. After disrupture of the tissue, the glucosinolates come into contact with thioglucosidase (also known by the trivial name myrosinase) and are converted into

glucose, sulfate and their corresponding isothiocyanates, thiocyanates and nitriles (Uda and Maeda, 1986; Springett and Adams, 1988). Thioglucosidase activity differs among the various crucifers (Springett and Adams, 1989; Palmieri et al., 1987; Wilkinson et al., 1984; Yen and Wei, 1993). Considerable variation in activity is also observed among cultivars of the same species and plant parts of specific cultivars (Pocock et al., 1987).

Extraction of glucosinolates from crucifer tissues is a difficult process, since glucosinolates are always accompanied by thioglucosidase and homogenization without inactivation of the enzyme always leads to a rapid loss in yield. For this reason many extraction protocols have been developed in which glucosinolates are extracted in various (boiling) organic solvents (Fenwick, 1984; Finnigan and Lewis, 1988; Hanley et al., 1983; McGregor et al., 1983; Peterka and Fenwick, 1988; Shasidi et al., 1990; Visentin et al., 1992) with the aim of inactivating thioglucosidase during the extraction process and to dissolve all glucosinolates with high recovery.

The diversity in glucosinolate content and composition in crucifers on the one hand and the isozyme patterns of thioglucosidase on the other hand suggest that glucosinolates play a dominant role in the flavor characteristics of cruciferous species. Glucosinolates and their corresponding breakdown products are indeed characterized as the main principles of aroma and flavor in cruciferous vegetables (Fenwick et al., 1983a). The content and distribution of glucosinolates have been studied in the edible parts of almost all species of commercial Brassica plants (Carlson et al., 1981; Daxenbichler et al., 1979; Heaney and Fenwick, 1980a, 1980b; Hill et al., 1987; Lewis and Fenwick, 1987, 1988; Sones et al., 1984; VanEtten et al., 1976, 1980) with the utilization of many different analytical techniques, which are reviewed by McGregor et al. (1983). More sophisticated techniques have since been developed for the determination of total glucosinolate content in samples: X-ray fluorescence (Schnug and Haneklaus, 1988), reflectance (Tholen et al., 1993), auto analyzer (Smith et al., 1985), soluble and immobilized enzymes (Kuan et al., 1986), or NIR spectroscopy (Biston et al., 1988). The determination of single glucosinolates has also been optimized;

e.g., glucosinolates now can be analyzed by high-performance liquid chromatography (HPLC) without desulfatation (Bjorkqvist and Hase, 1988). Indole glucosinolates can be determined by spectrophotometry after a simple derivatization step (Thies, 1990).

The determinations were, however, always restricted to a low number of cultivars, owing to time-consuming sample preparations and analysis of the glucosinolates by gas liquid chromatography (GLC) or high-performance liquid chromatography.

Enzyme-linked immunosorbent assays (ELISAs) are very suitable for the analysis of the glucosinolate content in large numbers of samples. ELISAs are particularly useful for the initial screening of the glucosinolate content and composition in hundreds of parental lines, cultivars and plants of segregating populations as present in breeding programs. For such a purpose conventional analytical techniques such as GC and HPLC are too time-consuming to allow an efficient selection during the selection season in plant breeding. An ELISA assay was published for the determination of alkenyl glucosinolates in series of seed samples by Hassan et al. (1988). The polyclonal antisera raised against a sinigrin-BSA conjugate showed a high cross-reactivity for progoitrin and gluconapin and were, therefore, not specific for the quantitative determination of sinigrin.

Particular Brussels sprout cultivars develop a pronounced bitter taste, which is evident in both fresh as well as frozen sprouts (Bedford, 1989), probably because of the presence of certain glucosinolates. The glucosinolate content of Brussels sprout cultivars consists mainly of sinigrin (allylglucosinolate), progoitrin (2-hydroxy-3-butenylglucosinolate), gluconapin (3-butenylglucosinolate) and minor amounts of indolglucosinolates (Carlson et al., 1987; Heaney and Fenwick, 1980a, 1980b; Heaney et al., 1983).

Fenwick et al. (1983a) and Griffiths and Fenwick (1984) found sinigrin and progoitrin to be the compounds that cause bitterness in buttons of Brussels sprouts. Sinigrin had a bitter taste as an intact glucosinolate, while progoitrin was intensely bitter after enzymatic decomposition to goitrin ((-)5-vinyloxazolidine-2-thione).

In order to study the influence of the (potentially) bitter glucosinolates on the taste preference of consumers and to select for a lower content in our breeding program, large scale convenient extraction methods and assays for glucosinolates were necessary.

In this paper, a simple sample preparation method for glucosinolates and specific ELISA assays for sinigrin and progoitrin are presented for the screening of breeding programs of Brussels sprouts for both glucosinolates.

Materials and methods

(A) ELISA assays for sinigrin and progoitrin. *Chemicals.* Radioimmunoassay-grade bovine serum albumin (BSA), ovalbumin (electrophoretic 99% pure), goat:antirabbit IgG-alkaline phosphatase conjugate (IgG-APase), Sigma 104 phosphatase substrate (PNP), N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, glucocheirolin and sinigrin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gluconapin and progoitrin were a kind gift of Dr. Brian Hanley and Dr. Roger Fenwick of the AFRC in Norwich, UK. Nunc class I plates were purchased from Nunc Inter-med., Copenhagen, Denmark. Standard reagents (pro analyze quality), which have been used in buffers, and organic solvents (purity, >99%) all were obtained from Sigma, Chemical Co. (St. Louis, MO).

Buffers. The coating buffer used was 100 mM sodium bicarbonate (pH = 9.6).

For phosphate buffered saline (PBS), 1 L of 150 mM PBS contained 8 g NaCl, 1.15 g $Na_2HPO_4:2H_2O$, 200 mg KCl and 200 mg KH_2PO_4, adjusted to pH = 7.2 with 2 N NaOH. PBS, pH = 7.6 was used as reaction buffer for the synthesis of sinigrin and progoitrin conjugates.

The washing buffer (PBST) was 0.05 % (v/v) (500 μL/L) Tween 20 in PBS.

The antibody diluent (PBSTO) was PBST buffer containing 0.01 % (w/v) ovalbumin.

The alkaline phosphate substrate consisted of 100 mM glycine, 1 mM $ZnCl_2$, 1.5 mM $MgCl_2:6H_2O$ and 1 mg/mL Sigma 104, pH = 9.7.

Synthesis of Sinigrin-BSA, Sinigrin-ovalbumin, Progoitrin-BSA and Progoitrin-Ovalbumin Conjugate. Sinigrin-BSA conjugate is prepared according to Hassan *et al.* (1988) with some modifications. Samples of 130 mg sinigrin and 152 mg succinic anhydride were dissolved in 1 mL dimethylformamide and 26 μL pyridin and allowed to react at room temperature for 5 days in a closed glass reaction tube.

The sinigrin hemisuccinate was crystallized by addition of 10 mL ether and collected by centrifugation (table centrifuge, 2000g). The precipitate was dissolved in 1 mL methanol and subsequently precipitated again by the addition of 10 mL diethylether and centrifuged (table centrifuge, 4000g). Thereafter, the precipitate was dissolved in 1 mL methanol and freeze-dried overnight.

The freeze-dried sinigrin hemisuccinate, 83 mg N,N'-dicyclohexylcarbodiimide (DCCD), and 90 mg N-hydroxysuccinimide were dissolved in 1.5 mL dimethylformamide in order to synthesize the activated N-hydroxysuccinimide ester of sinigrin. The solution was allowed to react for 12 h at 4 °C without any mixing. During this period, crystals of dicyclohexylurea were formed, which were removed by loading the solution on a disposable column equipped with a 4-μm porous glass filter followed by centrifugation (table centrifuge, 4000g). The clear filtrate (1.25 mL) was divided in two equal portions of 625 μL for the preparation of sinigrin-BSA and sinigrin-ovalbumin conjugates.

A quantity of 625 μL of solution of activated N-hydroxysuccinimide ester of sinigrin was added to either a solution of 21.2 mg BSA, or 42 mg ovalbumin in 2 mL PBS buffer pH = 7.6 and mixed gently for 24 h at 4 °C using a mechanical stirrer. In both mixtures a white sediment was formed immediately during the first hours. The crude sinigrin-BSA and sinigrin-ovalbumin conjugates were dialyzed 3 times for 12 h in dialysis tubing against 1 L demineralized water (Barnstead Nanopure apparatus, Boston, MA, USA) to remove unreacted chemicals. The dialyzed conjugates were freeze-dried and stored refrigerated (4 °C) in closed Eppendorf reaction vessels. The yield

was 42 mg sinigrin-BSA and 60 mg sinigrin-ovalbumin conjugate respectively.

The epitope density was determined using the difference in weight between the synthesized conjugate and the initial weight of BSA or ovalbumin. The following assumptions were made: molecular weight of BSA = 64 kD, free amino groups of BSA = 61, molecular weight of ovalbumin = 45 kD, free amino groups of ovalbumin = 20, molecular weight of sinigrin-moiety = 522.

The preparation of progoitrin conjugates was based on the same principle as for the sinigrin conjugates, but on a smaller scale. Samples of 50 mg progoitrin, 59 mg succinic anhydride, and 20 μL pyridine were incubated in 770 μL dimethylformamide for 5 days at room temperature. The isolation of the progoitrin hemisuccinate was identical to the isolation procedure of the sinigrin hemisuccinate. In contrast to the sinigrin hemisuccinate that had a white color, the progoitrin hemisuccinate had a typical brownish color. Samples of 52 mg of progoitrin hemisuccinate, 30 mg N-hydroxysuccinimide, and 27.6 mg of N,N'-dicyclohexylcarbodiimide were dissolved in 500 μL dimethylformamide and incubated overnight at 4 °C without any mixing. During the incubation, crystals of dicyclohexylurea were formed. The dicyclohexylurea was removed by centrifugation. The supernatant (750 μL) contained the active N-hydroxysuccinimide ester of progoitrin. A quantity of 185 μL was added to 21 mg ovalbumin dissolved in 1 mL PBS buffer, pH = 7.6. A quantity of 555 μL was added to 30 mg BSA dissolved in 3 mL PBS buffer, pH = 7.6. Both solutions were mixed gently with a mechanic stirrer for 24 h at 4 °C. The crude progoitrin conjugates were dialyzed three times for 12 h in dialysis tubing against 1 L demineralized water to remove unreacted chemicals, freeze-dried and stored refrigerated (4 °C) in closed Eppendorf vessels. Both conjugates had a typical brownish color.

NMR Spectroscopy on the Progoitrin Hemisuccinate. The glucosinolate moiety is coupled to BSA or ovalbumin by means of an active N-hydroxysuccinimide ester attached on the glucose molecule of the glucosinolate. Progoitrin not only contains hydroxyl groups on the glucose moiety, but also contains a hydroxyl group on C_3 of the aliphatic side chain. Esterification of

this specific hydroxyl-group will destroy the specific antigenic quality of the progoitrin molecule. NMR studies were done by Dr. C. Kruk, Department of Organic Chemistry at the University of Amsterdam to check whether the hydroxyl group of the progoitrin hemisuccinate was esterified. Two dimensional ^{13}C-^{1}H correlation NMR, Attached Proton Test (APT) ^{13}C NMR and proton NMR confirmed that the hydroxyl-group on C_3 of the aliphatic side-chain of progoitrin was not esterified.

Immunization of Rabbits. Two rabbits were immunized by Dakopatts (Glostrup, Denmark) with either the sinigrin-BSA or the progoitrin-BSA conjugate. The rabbits were immunized with 1 mL 0.1 M NaCl/Freund's complete adjuvant 1:1 (v/v) containing 1.32 mg of sinigrin-BSA or the progoitrin-BSA conjugate. Booster injections were applied every two weeks following the first immunization. The first bleeding was 68 days after the initial immunization. From every bleeding, a volume of 20-25 mL serum was obtained.

Thereafter, the rabbits were given a booster injection every 4 weeks and bled 12 days later. Thus, 3 bleedings of 20-25 mL antiserum were obtained from both rabbits. The sera were isolated from the blood by centrifugation and stored after the addition of 0.1% sodiumazide at -70 °C.

Coating, Titers, Specificity, Cross-Reactivity, Enzyme Assay. The coating of immunoplates, the determination of titers, specificity, and cross-reactivity of the obtained antisera, and the enzyme assay (alkaline phosphatase) were done according to standard procedures as described by Hassan *et al.* (1988). Cross-reactivity is defined as (sinigrin or progoitrin concentration for 50% inhibition)/(glucosinolate concentration for 50% inhibition) x 100.

(B) The extraction of glucosinolates with phosphoric acid. *Brussels Sprouts and Sample Preparation.* Hybrids of Brussels sprouts were grown under standard conditions in the trial field for cultivar assessment (for the evaluation of post-harvest traits such as uniformity, storage quality, flavor, etc.) as performed by Novartis Seeds BV in De Schermer, the Netherlands.

After they were harvested, duplicate samples of 200 gram were homogenized with 200 mL 4 v/v% phosphoric acid in a Braun

knife homogenator (Braun, Germany) for 1 min. Due to the 1:1 ratio of sprouts and 4% phosphoric acid (on a weight basis) is the effective phosphoric acid concentration during the homogenization step, 2%. After the homogenization step, the obtained slurry was transferred to a funnel that was positioned on a beaker of glass and equipped with a paper filter (Schleicher & Schuell, Germany) to separate the glucosinolate-containing fluid from solid cell-wall material. Of each obtained filtrate a duplicate sample of 1 mL was transferred to a 96-well plate (Micronic). Filled 96-well plates, containing 48 1-mL samples in duplicate, subsequently were centrifuged in a table centrifuge (Jouan, France, 4000g).

After centrifugation, 500 μL clear supernatant was transferred to a new 96-well plate and neutralized with approximately 55 μL 5 N KOH at pH = 7.0.

After neutralization the plates were centrifuged (Jouan, France, 4000g) to remove the calcium phosphate precipitate as sprouts were found to contain a high inorganic calcium content. The supernatant was transferred to a new microtiterplate, sealed, covered with a lid, and heated in a boiling water bath for 10 min to denature dissolved proteins and destroy putative residual enzyme activity which might recover after neutralization at pH = 7.0. Thereafter, the plates were cooled to room temperature by floating on water and again centrifuged to remove the precipitated sediments. Clear samples were stored in sealed microtiterplates at -20 °C until determination of the content of glucosinolates. By use of. this sample preparation protocol 100 samples could be prepared in duplicate per day.

Stability of Sinigrin and Progoitrin in 2% Phosphoric Acid and during Boiling. A good extraction solvent extracts glucosinolates efficiently and guarantees the stability of the glucosinolates during the extraction process. Phosphoric acid efficiently extracts amino acids, sugars and other water soluble metabolites from plant tissues. The stability of sinigrin and progoitrin in 2% phosphoric acid, the actual concentration during the extraction of glucosinolates, was studied in a time course of up to 4 h, a convenient period for the preparation of 100 sprout samples in duplicate. Sinigrin and progoitrin were dissolved at a

concentration of 2 mM in 5 mL 2% phosphoric acid or in 5 mL 350 mM potassium phosphate buffer (equimolar concentration phosphate), pH= 7.0 , as a control. Every hour a 500-μL sample was taken from the glucosinolate solutions and quickly neutralized at pH = 7.0 with 5 N KOH. A 2 mM concentration of glucose in 2% phosphoric acid and 350 mM potassium phosphate was included in the experiment as a positive and negative control.

The stability of the glucosinolates was determined in terms of split off glucose due to acidic hydrolysis using an enzymatic assay for glucose according to Boehringer Mannheim (1989) and expressed as percentage recovery of the initial concentration of 2 mM.

Phosphoric acid at a concentration of 2% effectively inactivates myrosinase and other enzymes during the extraction process but does not precipitate proteins as effective as perchloric acid or trichloroacetic acid (van Doorn et al., 1989). To precipitate inactivated but still dissolved proteins a cooking treatment of 10 min at 100 °C was found to be efficient. The stability of sinigrin and progoitrin was determined in samples of 2 mM of both glucosinolates in 2% phosphoric acid after neutralization and expressed as presented before.

(C) Determination of Sinigrin and Progoitrin in Sprout Samples. *Precision of the ELISA Assays.* Both ELISA assays for respectively sinigrin and progoitrin were tested for precision using an HPLC method for intact glucosinolates as a reference method (Bjorkqvist and Hase, 1988). Brussels sprouts samples were determined for the content of sinigrin and progoitrin with both ELISA assays and by HPLC.

HPLC Reference Method for the Determination of Glucosinolates. Samples of Brussels sprouts, as prepared with the phosphoric acid extraction method, were determined for the content of intact sinigrin and progoitrin by isocratic reversed-phase ion-pair chromatography. The HPLC system (Waters) was equipped with a C_{18} reversed-phase column and was run with an eluent that was composed of 100 mM ammonium acetate, 10 mM tetrabutylammonium chloride and 5% acetonitrile (pH = 7.0) at a flow of 2 mL/min. The glucosinolates were detected at 235 nm. The influence of the sample matrix on the % recovery of sinigrin

and progoitrin was studied with internal standards of both glucosinolates at a specific dilution of the samples. The recovery of both glucosinolates also was studied in a series of dilutions for specific samples.

Determination of the Total Sum of Glucosinolates. The total sum of glucosinolates was determined according to the modified glucose release method of Van Doorn et al. (1997).

Results and discussion

Breeding for secondary metabolites that have an impact on taste and quality of vegetables is often restricted by time consuming sample preparation and determination protocols.

In this report, the development of a relatively quick sample preparation method and two highly specific polyclonal antisera is reported that allows the large scale and quick determination of the glucosinolates sinigrin and progoitrin in the Brussels sprouts breeding program.

ELISA Assays for Sinigrin and Progoitrin.
Specific antibodies are a nice analytical tool for the quantitative determination of secondary metabolites such as glucosinolates. Antibodies are applicable for the screening of metabolites in samples by immuno assays. Antisera for immuno assays need to have sufficient specificity and a high titer. The prepared sinigrin-BSA and sinigrin-ovalbumin were very poorly soluble in the coating buffer. The poor solubility of the conjugates seem to be due to the high degree of conjugation since all free amino groups of the sinigrin-BSA and sinigrin-ovalbumin conjugates were occupied by the active ester of sinigrin. No protein was detected in the conjugates with an amino-group directed protein assay (data not shown). Immunization of two rabbits gave two different antisera with regard to the titer and specificity.

One of the two rabbits produced a highly specific antiserum against sinigrin as specified in this report. The antibody titer was determined with a dilution between 10^3- and 10^6-fold on plates coated with 50 ng sinigrin-ovalbumin/mL. The ^{10}log of dilution of antiserum and the extinction at 410 nanometer was linearly related at dilutions between 10^3- and 10^5-fold. The

antibody titer did not change during the three successive bleedings (not shown). Nonspecific background extinction was below 0.1 for all dilution's indicating the high selectivity of the antibodies for the sinigrin-ovalbumin conjugate.

Figure 1 shows the checkerboard titration of 4 different concentrations of sinigrin-ovalbumin antigen against 6 different dilutions of antiserum. A concentration of 50 ng sinigrin-ovalbumin/mL was the minimal quantity of antigen leading to an optimal extinction at all antiserum dilutions. 10^4-times-diluted antiserum gave one extinction unit within 2 h and was chosen to test the selectivity of the serum for sinigrin and the cross-reactivity for other glucosinolates.
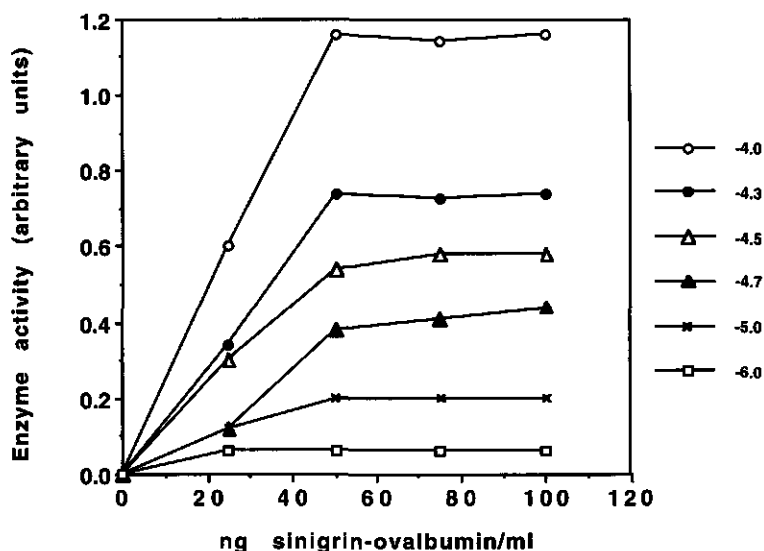
Figure 1. Checkerboard titration of four concentrations of sinigrin-ovalbumin antigen against six different dilutions of antiserum raised against sinigrin. Antisera dilutions are log transformed. Incubation time is 2 h at room temperature. Observed enzyme activity originates from alkaline phosphatase labeled mouse anti rabbit antiserum (see section Materials and Methods).

The typical standard curve for competitive inhibition of the sinigrin ELISA was log-linear between 10 nM and 10 μM sinigrin,

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an appreciable wide range to determine sinigrin concentrations in samples. The cross-reactivity of the antiserum against the glucosinolates gluconapin, progoitrin and glucocheirolin is shown in Figure 2. The antiserum turned out to be very specific for sinigrin, only gluconapin had a cross-reactivity of about 7.4%. The cross-reactivity of the glucosinolates progoitrin and glucocheirolin was negligible. The specificity of the antiserum for sinigrin increased from the first to the third bleeding, since the cross-reactivity for gluconapin decreased from 13.0 to 10.0 to 7.4% in the three successive bleedings, respectively.
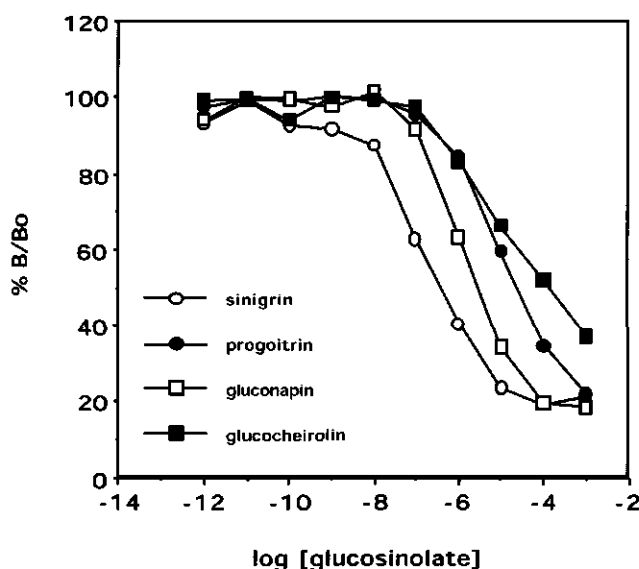
Figure 2. Competitive ELISA using anti-sinigrin serum. Plates were coated with 50 ng sinigrin-ovalbumin/mL and antiserum was 10^4-times-diluted. The glucosinolate concentration was varied between 1 pM and 1 mM for the glucosinolates sinigrin, progoitrin, gluconapin, and glucocheirolin. The incubation time was 2 h at room temperature.

The progoitrin-BSA and progoitrin-ovalbumin conjugate had the same properties as the sinigrin conjugates. All free amino groups in both conjugates were almost completely occupied by progoitrin moieties. After immunization of two rabbits, one rabbit raised a

highly specific antiserum against progoitrin. The titer of this serum was comparable with the titers obtained for the antisera against sinigrin. The second bleeding had almost the same titer. The rabbit died after the second bleeding.
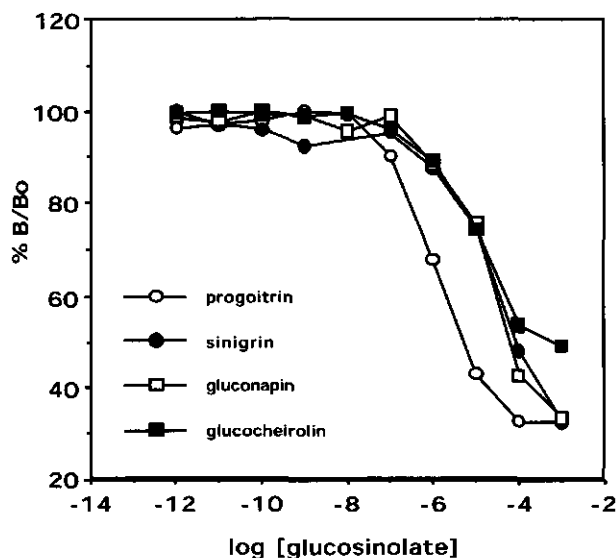
Figure 3. Competitive ELISA using the anti-progoitrin serum. Plates were coated with 50 ng progoitrin-ovalbumin/mL and anti-progoitrin serum was 10^4-times-diluted. The glucosinolate concentration was varied between 1 pM and 1 mM for the glucosinolates progoitrin, sinigrin, gluconapin, and glucocheirolin. The incubation time was 1 h at room temperature.

A checkerboard titration showed that plates were coated optimally with 50 ng progoitrin-ovalbumin/mL (not shown). Plates coated with 50 ng progoitrin-ovalbumin/mL and incubated with 10^4-times-diluted antiserum produced one extinction (410 nm) unit within 1 h. The background extinction was 0.3 (30% on the B/Bo scale), thus somewhat higher than for the sinigrin ELISA. The typical standard curve for the competitive inhibition of the progoitrin ELISA for the corresponding substrate progoitrin was log-linear between 10 nM and 100 μM progoitrin.

The cross reactivity of the antiserum against the glucosinolates sinigrin, gluconapin and glucocheirolin is shown in

Figure 3. The antiserum of the second bleeding had a cross-reactivity of 4.6% for sinigrin, gluconapin and glucocheirolin.

Hassan et al. (1988) reported antisera against sinigrin which had a high degree of cross-reactivity to other aliphatic glucosinolates. The first serum showed a cross-reactivity of 100% against the glucosinolates gluconapin and progoitrin. The second serum showed a cross-reactivity of 100 and 56% against, respectively, gluconapin and progoitrin. In contrast, our approach yielded highly specific antisera against sinigrin and progoitrin. In both cases, one of two rabbits immunized with sinigrin-BSA or progoitrin-BSA conjugate raised a highly specific polyclonal antiserum.

The sinigrin-BSA and sinigrin-ovalbumin conjugates of Hassan et al. (1988) were partially conjugated. Sixty-one and 65% of the free amino groups of BSA and ovalbumin, respectively, were occupied by sinigrin. Our conjugates were almost completely conjugated, since no free amino groups were detectable with amino-group-directed protein assays. This difference in conjugation rate might explain the higher specificity of our antiserum to respectively sinigrin and progoitrin. Coupled to this high specificity, the antisera show a very low cross-reactivity against other glucosinolates which might equally be the result of very uniform "high density" conjugates of respectively sinigrin and progoitrin-BSA.

The antiserum against sinigrin had a cross-reactivity of 7.4% for gluconapin, probably because gluconapin is very related to sinigrin, differing only in a $-CH_2$ group in the side chain of the glucosinolate.

Quality and Reliability of the Acidic-Sample Preparation Method. It is essential that the extraction process of glucosinolates is quick and efficient because of their rapid degradation in disrupted tissues (see Introduction). We developed a method in which 4 v/v% phosphoric acid is used as an extractant for the preparation of glucosinolate samples from Brussels sprout buttons. Sprouts and extractant were used in a 1:1 ratio during sample preparation resulting in an effective concentration of 2% phosphoric acid in the homogenate. The sample preparation method with phosphoric acid was tested for effectiveness at

three critical essential steps: (1) the acidic extraction step for the inactivation of thioglucosidase and the extraction of glucosinolates at room temperature, (2) the neutralization step to an optimal pH = 7.0 for glucosinolates, and (3) the boiling step to inactivate and remove residual proteins.

The stability of respectively 2 mM sinigrin and progoitrin was studied in 2% phosphoric acid, the actual concentration of phosphoric acid in the homogenate during the extraction step, for a period up to 4 h, a convenient time to prepare sprout samples on a large scale. Both glucosinolates were found to be stable in this acidic environment during the whole period. A recovery of 99 $\pm$1% (n = 2) was observed after determination of the glucosinolates by means of the glucose-release method of Van Doorn et al. (1997). Boiling of neutralized 2 mM sinigrin and progoitrin solutions for a period of 10 min gave the same recovery for both glucosinolates (data not shown).

Within Brussels sprout samples, a sinigrin and progoitrin content of maximally 6 and 4 g/kg fresh weight, respectively, have been found (van Doorn et al., 1997), equivalent to concentrations of up to 10 mM of the individual glucosinolates in neutralized sprouts samples according to our extraction protocol.

An internal standard of 2 mM sinigrin and progoitrin was added to samples of sprout buttons during the homogenization step with phosphoric acid, and afterwards the recovery of both glucosinolates was determined by an assay for the total glucosinolate content by the glucose release assay. The results indicated that sinigrin and progoitrin were stable in the acidic matrix during the extraction process (data not shown).

From the results it can be concluded that sinigrin and progoitrin can be effectively extracted from fresh sprout samples without any degradation of these glucosinolates during the successive sample preparation steps; e.g., the homogenization in 2% phosphoric acid, the neutralization with KOH, and the boiling to remove proteins. This method, therefore, was applied for the large scale preparation of sprout samples.

The method yields samples that contain a sinigrin and progoitrin content that exceed the values reported previously (Fenwick et al., 1983b; Heaney et al., 1983) which might be

indicative that the phosphoric acid extraction procedure has a better recovery of glucosinolates. However, new, improved cultivars have been introduced on the market since then, which is an alternative explanation for the increased content of sinigrin and progoitrin of cultivars in this study. The extraction protocol has clear advantages in comparison to the currently used methods in which glucosinolates are extracted in boiling organic solvents. The acidic inactivation of myrosinase allows the large scale preparation of samples with relatively simple homogenators. Time consuming steps such as vacuum evaporation, the addition of internal standards and quantitative collection of glucosinolates are not necessary in our protocol.

Determination of Intact Sinigrin and Progoitrin by HPLC.
The determination of glucosinolates by HPLC is routinely conducted on glucosinolate samples that have been obtained by classical isolation methods involving boiling with organic solvents. Such samples contain glucosinolates in a relatively clean matrix in which also other organic solvent soluble compounds are dissolved. In the case of analysis of desulfoglucosinolates, the matrix of the samples is even more free of disturbing plant metabolites, since the matrix is then only composed of sulfatase in an acetic acid buffer. The influence of the complex sample matrix, as obtained after the phosphoric acid extraction procedure, on the determination of glucosinolates by isocratic ion-pair reversed phase HPLC was tested (see Materials and Methods). Progoitrin and sinigrin elute from the column after 1.5 and 2.0 min, respectively, under the specified conditions.

The peak area for sinigrin and progoitrin are found to be highly linearly related to the injected sample volume for 100-times-diluted samples. Linear relationships ($n = 3$, $r^2 > 0.99$) were observed between the sample volume and the peak area for up to 200 μL of injected sample with an intercept for the lines close to zero. Comparable figures were obtained at other dilutions. It was concluded that the sample matrix had no influence on the quantification of both glucosinolates using ion-pair HPLC.

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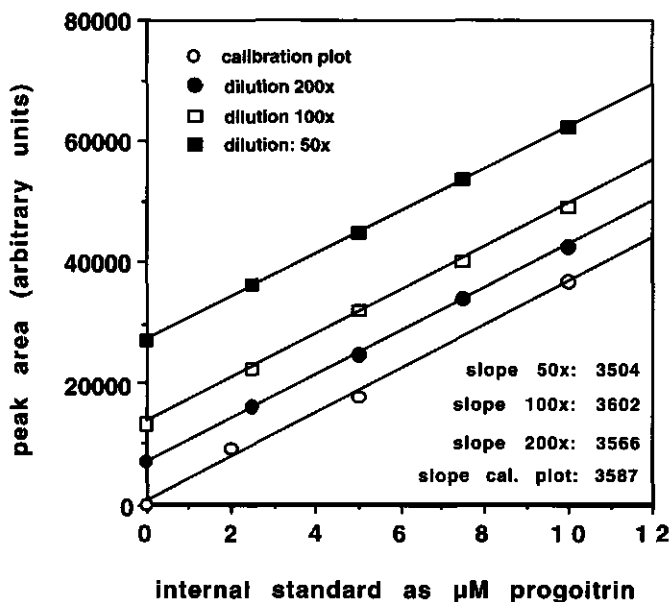
Figure 4. Recovery of internal standards of progoitrin in 100-fold-diluted sprout samples. Internal standards were added as a concentration of 0, 2 and 6 μM glucosinolate. Peak area is given in arbitrary units of the detector at 0.002 AUFS.

In Figure 4, four 100-times-diluted sprout samples are spiked with an internal standard of 0, 2 and 6 μM of progoitrin. The figure clearly shows that the different samples and the concentration of progoitrin in the samples had no influence on the recovery of the internal standards. The slopes that were calculated from the relationships between the concentration of internal standard and the glucosinolate concentration in the spiked samples were almost equal to the slope of the calibration plot of the internal standard in water. The average slope of samples was only 2.8% higher than the slope of the calibration plot. Comparable recovery figures were obtained when sinigrin was spiked in these four samples (data not shown).
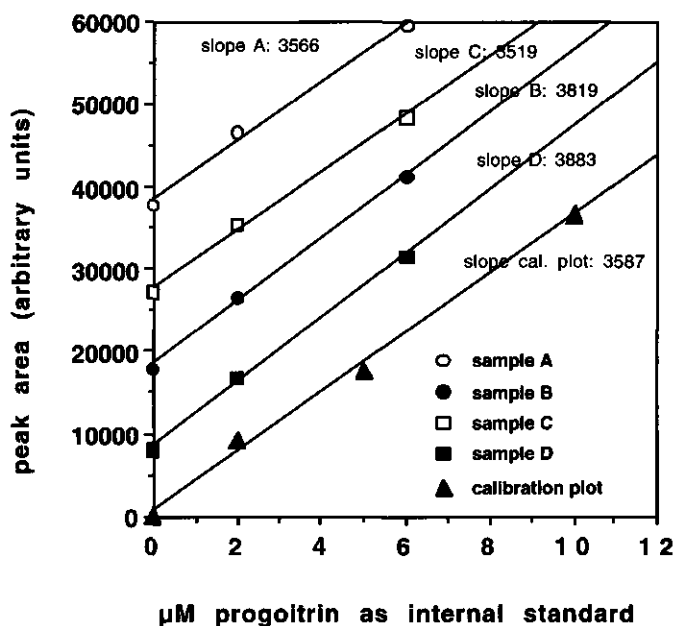
Figure 5. Recovery of internal standards of progoitrin in sprout samples at variable dilutions. Internal standards were added as a concentration of 0, 2.5, 5.0, 7.5 and 10 μM glucosinolate. Peak area is given in arbitrary units of the detector at 0.002 AUFS.

In Figure 5, a sample is diluted 50, 100 and 200 times and spiked with 0, 2.5, 5, 7.5 and 10 μM progoitrin, respectively, to study whether the dilution rate has an influence on the recovery of added internal standard. The recovery of the spiked internal standards, expressed as the slope from the relationship between the concentration of the internal standard and the glucosinolate content in the spiked samples, is on average almost 100% for progoitrin (-0.8%). Again, comparable recovery figures were obtained when sinigrin was spiked in samples with a variable dilution rate (data not shown).

From the results it is clear that samples, which have been prepared with the phosphoric acid extraction protocol, can easily be determined with ion-pair RP HPLC. The content of progoitrin and sinigrin is linearly related to the dilution rate, internal

standards show 100% recovery at variable sample dilutions and at variable concentrations of both glucosinolates.

Sinigrin and Progoitrin Content of Hybrids of Brussels sprouts: A Comparison between the ELISA Assays and the HPLC Method. The antisera were applied on samples of sprouts from various cultivars that have been prepared by phosphoric acid extraction method. The samples were also determined for the content of both glucosinolates by the HPLC method to compare the methods with each other. The sinigrin and progoitrin content of samples could be determined with the ELISA assays using a dilution factor of 1000 to obtain concentrations of sinigrin and progoitrin in the linear concentration range of the calibration plot.

In Figure 6 the sinigrin content in samples of 12 different cultivars as determined by HPLC and ELISA was correlated (top graph). It is shown that the sample preparation method yields samples that contain a sinigrin content from 40 to 250 mg/100g fresh weight. From the equation that describes the relationship between the content determined by HPLC and antibody assay it is clear that both methods are almost comparable for aspects such as sensitivity (slope = 0.95) and reliability (r^2 = 0.92). Both methods will yield about the same sinigrin content in samples.

In the lower graph of Figure 6 the progoitrin content of the same samples as determined by ELISA and HPLC is correlated. The highest progoitrin content in the samples was, depending on the method, about 90 or 130 mg/100g fresh weight.

The equation that describes the relationship between both methods shows that the ELISA assay for progoitrin in all cases gives a higher progoitrin content in samples than the HPLC method. The progoitrin content as obtained with either the ELISA or HPLC method is relatively very reliable (r^2 = 0.92, n = 12, p < 0.01).

The higher progoitrin content in the ELISA assay might be due to a matrix effect of the samples. Progoitrin is a relatively polar glucosinolate which is hydroxylated on the alkyl-group. A polar matrix, which has, for instance, a high phosphate concentration, might decrease the binding of antibodies to the coated progoitrin-ovalbumin conjugate during the competitive binding step of the

ELISA assay. Thus, an overestimation of the progoitrin content occurs which is based on an artifact. The overestimation of the progoitrin content in the ELISA assay also might be explained by the quality of the polyclonal antiserum. The possibility cannot be excluded that (part of) the polyclonal progoitrin-specific antibodies bind to an epitope which is composed of two conjugated progoitrin molecules.

The observed difference between the ELISA and HPLC method is about 50% but proportionally comparable for samples that differ in the content of progoitrin, allowing the correction for this phenomenon by extrapolation.

Time-consuming glucosinolate determinations by HPLC can be replaced by rapid, reliable, and quantitative ELISA assays. With ELISA assays, the number of samples no longer forms a limitation to the determination of glucosinolates. Both antisera proved to be perfect tools for the determination of sinigrin and progoitrin content in edible parts of cruciferous vegetables and can also be applied for the determination of these glucosinolates in other types of samples such as seeds. The application of specific antisera for the analysis of glucosinolates is of significant importance to the study of glucosinolate metabolism and inheritance in breeding programs of Brassica vegetables.
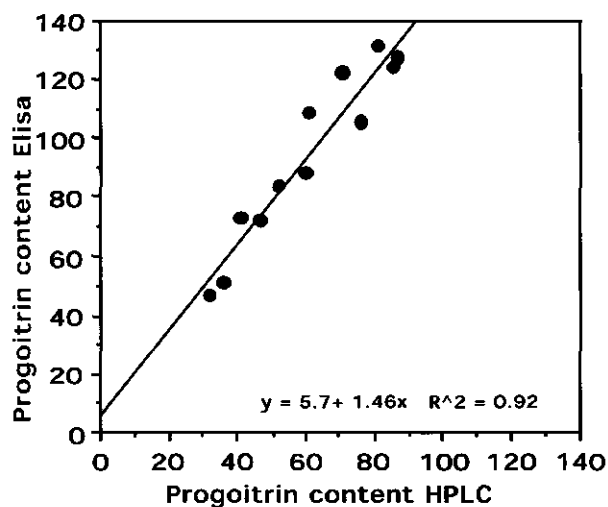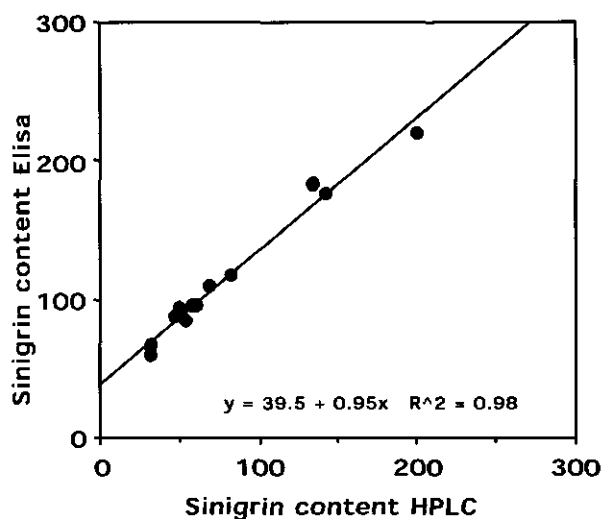
Acknowledgment

Figure 6. Correlative studies to compare HPLC and ELISA assays for the glucosinolates sinigrin (upper graph) and progoitrin (lower graph). The determination of the content of both glucosinolates was conducted as described under Materials and Methods. The content of both glucosinolates is expressed in mg/100g.

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Chapter 3
Large scale determination of glucosinolates in Brussels sprouts samples after degradation of endogenous glucose.

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Abstract

A method was developed for the determination of the glucosinolate content in glucose-rich samples of Brassica vegetables such as Brussels sprouts. Glucose in the samples was enzymatically degraded by the enzyme glucose oxidase (GOD). The resulting hydrogen peroxide and the enzyme GOD were thereafter respectively dissociated and inactivated by a heat treatment at 100 °C. After the degradation of endogenous glucose the glucosinolates were converted into glucose and related metabolites with the enzyme thioglucosidase originating from Brussels sprouts seeds. Glucose released was determined enzymatically with a glucose oxidase/peroxidase assay as a measure for the glucosinolate content of samples. The method was used to study the influence of harvest time, crop production location and the choice of parental lines on the glucosinolate content of Brussels sprouts F1-hybrids. The sum of sinigrin and progoitrin of F1-hybrids was found to be significantly correlated to the glucosinolate content.

Introduction

Glucosinolates are thioglycosides which are present in more than 90 forms in cruciferous species (McGregor et al., 1983). In Brussels sprouts, a high content of the glucosinolates sinigrin and/or progoitrin (Fenwick et al., 1983; Griffiths and Fenwick, 1984) is correlated with bitterness and a low preference score by consumers (Van Doorn et al., 1998a).

Therefore, breeding for a low content of both glucosinolates in Brussels sprouts is preferred for the development of cultivars with a satisfactory flavor for consumers. In recent years, specific ELISA assays have been developed for the determination of sinigrin and progoitrin in Brussels sprouts (Van Doorn et al., 1998b). These assays were applied to support selection for a low sinigrin and progoitrin content in parental lines, inbred populations and (experimental) F1-hybrids. Newly developed assays, such as ELISAs, have to be compared with the available reference methods (McGregor et al., 1983) to check their

reliability, accuracy and sensitivity. Individual intact glucosinolates are determined routinely by means of HPLC (Bjorkqvist and Hase, 1988). The total glucosinolate content of samples is usually determined via anion exchange chromatography, the subsequent glucose release step with the glucosinolate degrading enzyme thioglucosidase (E.C. 3.2.3.1) and finally the determination of glucose (VanEtten and Daxenbichler, 1977). The assay for the glucosinolate content is not only an essential reference method for the recovery of glucosinolates but is also relevant for the calculation of the relative content of sinigrin and progoitrin in parental lines and F1-hybrids.

The currently used protocols are too complicated to allow the analysis of large numbers of samples. The time-consuming chromatography step is, however, essential in the currently used method to remove the glucose already present in samples which dominates the released glucose from glucosinolates in the glucose assay.

Various alternative methods have been developed that directly (Thies, 1982) or indirectly (Schnug, 1987) determine the total glucosinolate content of seed samples. The method of Thies, based on complexation of glucosinolates with tetrachloropalladate, is only applicable to seed samples as the matrix of samples from other plant parts of Brassica crops seriously interferes in the assay. The sulfate release method of Schnug also works well on seed samples but the sulfate release from glucosinolates by the enzyme sulfatase is significantly inhibited at a phosphate concentration higher than 100 mM. The determination of the total glucosinolate content of e.g. seed samples, has been further optimized by Smith et al. (1985) and Tholen et al. (1993), who respectively applied an auto analyzer and a TRUBLUGLU meter for relatively large scale determinations. However, seed samples contain a glucose content below 1 g/100g which does not interfere with the determination of released glucose in the total glucosinolate assay. Therefore, the methods are not applicable on samples of edible parts from the various Brassica species, as these have a sugar content of up to 8 grams per kilogram fresh weight.

In this paper, the glucose degradation of samples with a high glucose content and the subsequent determination of the glucosinolate content is presented. The method is applied to study the influence of crop production location, harvest time at specific sites, and the choice of parental lines on the glucosinolate content of Brussels sprouts cultivars.

Materials and Methods

Chemicals and enzymes. Sinigrin, phenol, aminophenazon, lead acetate, barium acetate, standard reagents (pro analyze quality), which have been used in buffers and organic solvents (purity >99%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucose oxidase (GOD) and peroxidase (POD) were obtained from Boehringer Mannheim (Mannheim, Germany).

Preparation of samples. Samples were essentially prepared according to the method of van Doorn et al. (1998b) with some modifications. Samples from Brussels sprout lines and F1-hybrids were obtained from various sites of the Novartis Seeds BV breeding program in the Netherlands. In summary, the Brussels sprouts samples were homogenated in duplicate with 4 % (v/v) phosphoric acid, filtered on a paper filter and a clear supernatant was obtained by centrifugation. The supernatant was neutralized with 5N KOH at pH 7.0 and precipitates were removed by centrifugation. The clear supernatant was heated in a boiling water bath for 10 min and cooled to room temperature. Subsequently, the samples were additionally treated with 0.5 M lead and barium acetate. After centrifugation, the clear samples were stored at -20 °C, ready for the determination of the glucosinolate content. In all experiments presented in this paper, the glucosinolate content of samples was determined within 3 months after sample preparation. Measurements revealed that the glucosinolate content of frozen samples at -20 °C was constant for a period of one year.

Isolation of thioglucosidase from Brussels sprout seeds. Thioglucosidase was, for economic reasons, isolated on a large scale from Brussels sprout seeds according to the method of Smith et al. (1985) with some modifications. Seeds from the

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Brussels sprout cultivar Lauris (Novartis Seeds BV) were homogenated in an electric coffee mill and defatted with hexane. Twenty grams of flour was vigourously extracted with 100 mL hexane in triplicate and the sediments were subsequently left to dry overnight on filter paper. All purification and isolation steps for thioglucosidase were conducted in the cold room at 4 °C. The defatted flour (20 g) was extracted with 100 mL 30 % (v/v) ice-cold acetone for 30 min. The slurry was centrifuged (Sorvall RC-5B, 13000 rpm, 15 min, 4 °C). The pellet obtained was re-extracted with 50 mL 30 % (v/v) of ice-cold acetone under the same conditions. The second supernatant, obtained after centrifugation, was added to the first supernatant and the acetone concentration was increased dropwise to 70 % (v/v). The precipitate was collected by centrifugation (13000 rpm, 15 min, 4 °C) and dissolved in a small volume of demineralized water (Barnstead Nanopure, Boston, MA). The dissolved protein was transferred to dialysis tubing and dialyzed for 4 h against, respectively, 2 L 10 g/L NaCl, 5 g/L NaCl and water. The dialysis tubing, containing the enzyme thioglucosidase, was centrifuged (13000 rpm, 15 min, 4 °C) to remove precipitates, lyophilized and stored at 4 °C in closed storage tubes until utilized in the glucosinolate assay. The yield was about 300 mg crude thioglucosidase protein. The specific activity of the crude thioglucosidase was determined with sinigrin as substrate prior to its utilization for the glucosinolate determination described later in this section. The lyophilized enzyme was used in the glucosinolate determination at a concentration of 10 mg/mL in 100 mM sodium phosphate buffer pH 7.0. The enzyme solution was centrifuged (Eppendorf table centrifuge 10000g, room temperature) prior to utilization in the assay. The enzyme solution for the assay was prepared fresh each time to ensure optimal activity.

Determination of thioglucosidase activity in the enzyme preparation. Thioglucosidase activity in the crude preparation was determined as the rate of glucose released from sinigrin in 100 mM sodium phosphate buffer pH 7.0 by 50 μL of the enzyme stock solution (10 mg/mL) and by 100 μL of, respectively, 1.2 and 2 mM sinigrin per mL assay buffer. Glucose release, as parameter

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for thioglucosidase activity, was monitored from 0 to 75 min at intervals of 5 min. The thioglucosidase activity assay was conducted in a reaction mixture of 5 mL. Two hundred microliter subsamples were taken from the reaction reservoir. The thioglucosidase activity in the sub samples was directly inactivated by exposure of the samples for a period of 5 min to 100 ºC in a water bath. The heated samples were rapidly cooled on ice to room temperature, centrifuged to remove protein precipitations, and determined enzymatically for released glucose. Incubations with the standard sinigrin concentrations in the absence of thioglucosidase served as a control. The glucose content of the samples was determined in a 100 mM sodium phosphate buffer pH 7.0 containing phenol (1 mg/mL), GOD (65 U/mL), POD (5 U/mL) and aminophenazon (15.6 mg/100 mL) according to the method of Boehringer Mannheim (1989). In this assay, glucose is converted into a complex with a red color which can be determined at 520 nm by the use of a microtiter plate reader. Color development was determined 1 h after the start of the glucose assay.

Degradation of glucose in glucosinolate containing Brussels sprout samples. The degradation of glucose was conducted in 96-well microtiter plates (Greiner, The Netherlands) in a 100 mM sodium phosphate buffer (pH 7.0) containing 65 U/mL GOD. A volume of 190 μL GOD/phosphate buffer was mixed with 10 μL sample and incubated for 4 h at room temperature. During the degradation period the plates were continuously mixed on a microplate mixer to assure that sufficient oxygen was available for the oxidation of glucose by GOD. After 4 h, the microtiter plates were sealed and heated for 10 min in a water bath of 100 ºC which was just not boiling. Thus, the formed hydrogen peroxide was dissociated and the enzyme GOD inactivated. After cooling, the glucose-free samples were used for the determination of the glucosinolate content after a centrifugation step for the collection of adhering condensation on the seals.

Determination of the glucosinolate content in Brussels sprout samples. Ten microliters 10 mg/mL thioglucosidase solution in 100 mM sodium phosphate buffer pH 7.0 was added to glucose-free samples, as prepared according to the previous section. As a

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blank, counter samples were incubated in absence of enzyme. Calibration solutions (0, 2, 4, 6, 8 and 10 mM sinigrin) were used to check the thioglucosidase activity across a broad concentration range. After mixing, the plates were sealed and the glucosinolates were degraded overnight at room temperature. The plates were then centrifuged (IEC Centra 4B with microtiterplate adapters, 4000 rpm, 10 min, room temperature) to remove minor precipitations originating from the thioglucosidase solution. Released glucose from glucosinolates was determined in 100 mM sodium phosphate buffer pH = 7.0 containing phenol (1 mg/mL), GOD (65 U/mL), POD (5 U/mL) and aminophenazon (15.6 mg/100 mL), the glucose assay buffer. A volume of 100 μL of thioglucosidase treated sample was added to 100 μL of glucose assay buffer. After mixing, the samples were incubated for 1 h at room temperature in darkness and red color formation was subsequently determined in a microtiterplate reader (Biotek, USA) at a wavelength of 520 nm and a reference wavelength of 600 nm. Glucose calibration solutions (100 μL) (0, 0.08, 0.16, 0.24, 0.32 and 0.40 mM) in 100 μL glucose assay buffer were used to calculate the concentration of released glucose from glucosinolates in the samples. In the calculations, an average glucosinolate molecular weight of 457 was used (Carlson et al., 1987). The glucosinolate content of samples was expressed as mg/100g fresh weight. The glucosinolate content of samples was determined in duplicate, and only duplicate readings differing 0.020 absorbance unit or less were used for calculations.

The glucosinolate content of Brussels sprout lines during development. To study the influence of sprout button maturity on the glucosinolate content sprouts from 12 lines were harvested at 4 monthly intervals starting in October 1989. Samples of 200 gram per line were collected on October 15, November 15, December 15 and January 15 in the season 1989-1990. Sprout samples were obtained from the assessment trials, as executed by Novartis Seeds BV for commercial F1-hybrids in De Schermer (north Netherlands on loam soil).

The glucosinolate content of F1-hybrids at various locations. To study the influence of crop production site on the glucosinolate content 30 sprouts F1-hybrids were grown in 1989 in two

different trial fields of Novartis Seeds BV in the Netherlands. The F1-hybrids were grown in, respectively, De Schermer (north Netherlands on loam soil) and in Barendrecht (midwest Netherlands on loam soil). Sprouts of each cultivar were harvested simultaneously at the two locations from October to November, the period for an optimal glucosinolate content, and determined for the glucosinolate content. Sprouts of each cultivar were harvested at their optimal harvest time with regard to consumption quality.

The proportion of sinigrin and progoitrin in the glucosinolate content. The samples of the former paragraph also were determined for the content of sinigrin and progoitrin according to Van Doorn et al. (1998b) to define the proportion of these glucosinolates in the glucosinolate content. The sinigrin and progoitrin content of samples was determined in duplicate, and only duplicate readings differing 0.020 absorbance unit or less were used for calculations.

The glucosinolate content of F1-hybrids and corresponding parental lines. In 1989, 150 experimental F1-hybrids and their approximately 50 corresponding parent lines were grown on the trial field of Novartis Seeds BV in De Schermer, in north Netherlands, on loam soil. The F1-hybrids and parental lines were grown under standard conditions and sprouts of both the hybrids and their corresponding parent lines were harvested at their optimal harvest time with regard to consumption quality and their glucosinolate content determined.

Results and Discussion

Isolation and characterization of thioglucosidase from Brussels sprouts seeds. Brassica seeds contain a high content and activity of the enzyme thioglucosidase. The former enzyme can be applied for the determination of the glucosinolate content in Brassica samples by means of glucose release (Smith et al., 1985).

Thioglucosidase was isolated from seeds of the Brussels sprout cultivar Lauris. The thioglucosidase enzyme from Brussels sprout seeds showed a similar behavior in the subsequent isolation steps

as reported for the *Brassica napus* enzyme (Smith et al. (1985). The thioglucosidase from Lauris seeds was tested for activity using sinigrin as substrate. Figure 1 shows the rate of glucose release from sinigrin by the enzyme preparation. The enzyme concentration in the assay was chosen to ensure a complete glucose release from glucosinolates within 1 h.

At the specified enzyme concentration, sinigrin was degraded to glucose in about 30 min at both sinigrin concentrations. The observed absorbance values at the end of the activity assay, 0.3 and 0.5 for respectively 1.2 and 2.0 mM sinigrin, are equal to the values observed with glucose concentrations of respectively 1.2 and 2 mM in the glucose assay. No glucose release was detected from sinigrin during assay in the absence of thioglucosidase (data not shown). The thioglucosidase preparation was used for the determination of the glucosinolate content with a concentration of 50 μl 10 mg/mL enzyme per mL assay buffer. The thioglucosidase preparation should not contain other glucose producing enzymes such as sucrose invertase, because glucose production via side reactions will lead to an overestimation of the glucosinolate content in samples. The preparation was essentially free from sucrose invertase activity as no degradation of sucrose to glucose and fructose was observed in the presence of the enzyme (data not shown). Tholen et al. (1993) have clearly indicated that Brassica seeds are, apart from thioglucosidase, free from glucose producing enzymes other than sucrose invertase. Their determinations of the seed glucosinolate content by either desulphoglucosinolates and HPLC or by autolysis of glucosinolates with endogenous thioglucosidase, comparable to our method, gave exactly the same results in seven seed samples which varied a 6-fold in glucosinolate content. Brussels sprouts contain, besides glucosinolates and sugars, the glucose containing polymers (hemi)cellulose and starch in amounts that might disturb the glucosinolate assay. The acidic extraction protocol in combination with centrifuge steps at 10.000 x g excluded the presence of these compounds in samples (van Doorn et al., 1998b).
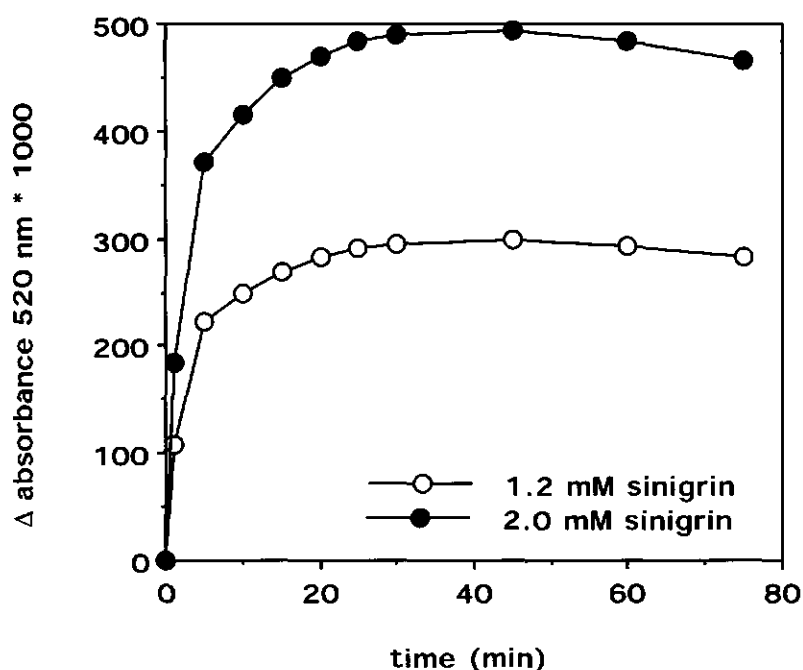
Figure 1. Thioglucosidase activity of a crude enzyme preparation from seeds of the Brussels sprout cultivar Lauris. Glucose release is determined by the GOD/POD assay.

Table 1. Mean glucose and glucosinolate content of Brussels sprout test samples used for the development of the glucosinolate assay with sugar-containing samples. The samples were determined for the content of glucose and glucosinolates in duplicate.

sample	glucose (g/100g)	glucosinolates (mg/100g)
1	0.6	260
2	1.1	310
3	2.5	190
4	1.4	190
5	1.1	300
6	1.2	300
7	0.7	260
8	0.5	510
9	0.7	320
10	0.6	280

Degradation of glucose to gluconate and hydrogenperoxide by GOD. The absence of glucose at the start of the assay is essential for a proper determination of the glucosinolate content of samples via thioglucosidase mediated glucose release. In standard protocols (e.g. VanEtten and Daxenbichler, 1977) samples are freed from sugars by ion-exchange chromatography. Glucosinolates are bound to anion exchange resins by means of their sulfate group, while glucose and many other compounds have no retention and can easily be washed from the column. The glucosinolates, however, only bind to anion exchange resins at a relatively low ion strength which means that the extraction of glucosinolates has to be conducted with water, low ionic strength buffers or polar organic solvents such as methanol or ethanol. The extraction of glucosinolates with organic solvents is slow and comprises a sample homogenization step, an extraction step with reflux of the extraction solvent and finally an evaporation step. In a previous paper, Van Doorn et al. (1998b) have presented a new extraction protocol that allows the extraction of glucosinolates with 4% phosphoric acid on a large scale. Samples prepared with this new protocol contain a phosphate concentration of 350 mM, which is too high for the separation of glucose from glucosinolates by means of ion exchange chromatography. The glucose has to be removed by an alternative method from glucosinolate containing samples in a matrix of 350 mM phosphate. Glucosinolate containing samples can be freed from glucose by its degradation with the enzyme GOD as an alternative to ion exchange chromatography. The enzyme GOD converts glucose to gluconate and hydrogen peroxide while the glucosinolates remain intact. The oxidation of glucose in glucosinolate containing samples is a nonequilibrium reaction that continues until all glucose is oxidized to gluconate and hydrogen peroxide providing that sufficient oxygen is available (Boehringer Mannheim, 1989). In the glucose degradation step, the resulting hydrogen peroxide can be removed by heating, which simultaneously inactivates the GOD. The glucose content of 10 test samples, with maximum variation of glucose content, was determined with the GOD/POD assay and varied between 0.5 and 2.5 g/100g fresh weight (Table 1). A

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glucose content of 2.5 g/100g in sprouts corresponds with a glucose concentration of approximately 58 mM glucose in sprout samples as prepared by the method of van Doorn et al. (1998b). In the glucose degradation step, the samples were diluted a further 20 times in GOD/phosphate buffer. The highest glucose concentration (3 mM) was degraded within a period of 4 h. The degradation products in the reaction seemed to have no influence on the conversion of glucose into its degradation products at these concentrations. The power of the degradation protocol is based on the lability of hydrogen peroxide, which is completely degraded into water and oxygen by a heat treatment.

The glucose degradation protocol can be conducted with series of microtiter plates on a much larger scale in comparison to any of the anion exchange protocols (Kuan et al, 1986; McGregor, 1985). An additional advantage of the protocol is that the matrix of the glucose-free samples almost resembles the composition of the buffer which is used in the enzymatic standard protocols for the analysis of glucose (Boehringer Mannheim, 1989; Palmieri et al., 1987).

Glucose release from glucosinolates with the enzyme thioglucosidase. The overnight degradation of glucosinolates in the 10 glucose-free samples by thioglucosidase resulted in glucose contents which were equivalent to a glucosinolate content between 190 and 510 mg/100g fresh weight (Table 1). The cultivars differed significantly for the content of glucosinolates (factorial ANOVA, $p = 0.001$, LSD = 57). The values observed are in agreement with, or sometimes even higher than the presented total glucosinolate figures for Brussels sprout lines and cultivars in the literature (Heaney and Fenwick, 1980; Heaney et al., 1983; Carlson et al., 1987). The glucosinolate assay with an enzymatic glucose degradation step apparently gives realistic glucosinolate figures for samples from sprout lines and F1-hybrids.

The overnight degradation of glucosinolates in glucose free samples was complete, as no intact glucosinolates were detectable by HPLC analysis (data not shown) after the degradation step using the method of Bjorkqvist and Hase (1988).

As expected, the glucose and glucosinolate content of the samples were not correlated ($r = 0.57$, $n = 10$, $p > 0.05$).

The influence of the sample matrix on the glucosinolate determination. According to the law of Lambert-Beer the absorbance of an ideal enzymatic end-point assay is a log-linear function of the concentration of the analyte. Log-linear relationships between the concentration of analyte and the absorbance can be obtained by variable concentrations of analyte in a fixed matrix or by variable volumes of a matrix with a fixed concentration of analyte. At variable sample volumes in the glucose end-point assay, a linear relationship was observed between the sample volume and the absorbance at the end of the assay (Figure 2). The sample matrix apparently has no influence on the complete conversion of glucose to a red coloured complex in the assay. In case of inhibition of the glucose conversion into the red complex by specific factors in the matrix, a typical saturation curve would have been observed between the sample volume and the absorbance values. A sample volume of 100 μL was chosen as a compromise for sample volume, sensitivity and the maximal absorbance increase in the assay.

The glucosinolate content of Brussels sprout lines during development. The glucosinolate assay can be used to study the influence of harvest time on the glucosinolate content of Brussels sprout lines and cultivars. The glucosinolate assay was used to study the glucosinolate content of 12 lines in consecutive harvests between October and January. Figure 3 shows the glucosinolate content of a selection of lines and the average content of all lines. Considerable variation in glucosinolate content is observed for consecutive harvests, an indication that the content of cultivars depends on both genetics and the maturity of the sprouts at the time of harvest.

The glucosinolate content of specific lines has an optimum in harvest time. Some lines already start with the highest glucosinolate content in October and decrease in content. Other lines show the highest content in November/December and then decrease in content later.

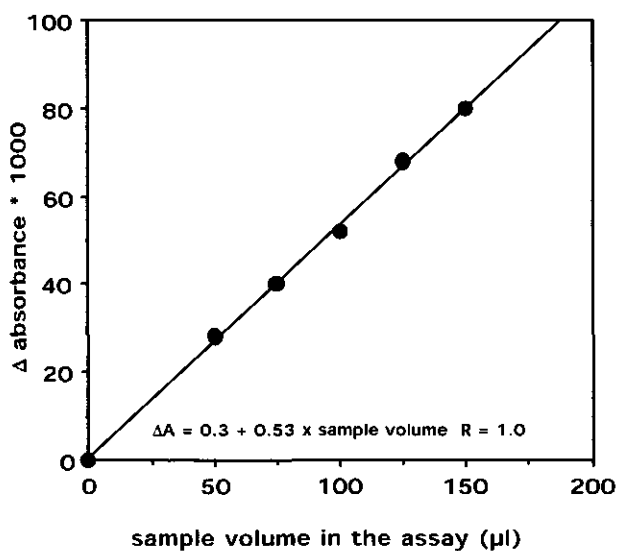$\Delta A = 0.3 + 0.53 \times \text{sample volume} \quad R = 1.0$

Figure 2. Determination of released glucose from glucosinolates at variable sample volumes in the glucose assay.
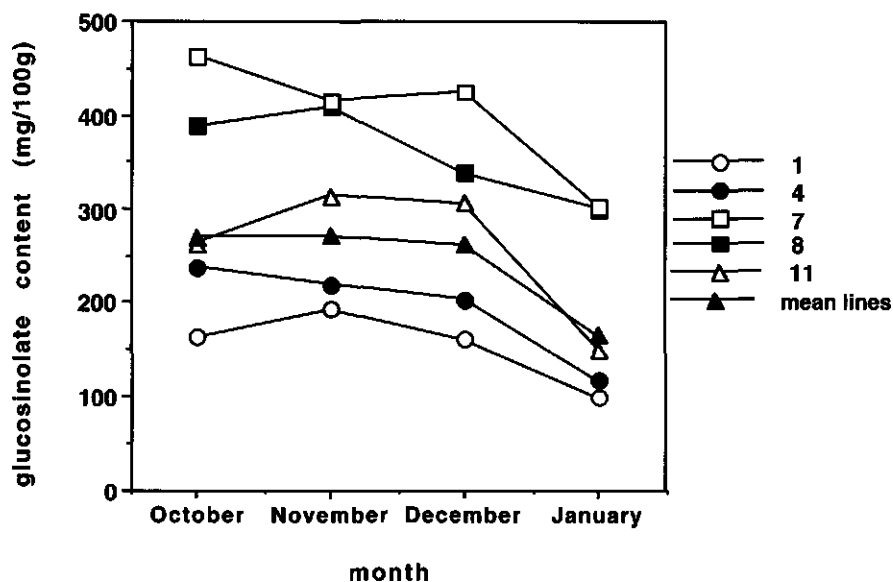
Figure 3. Glucosinolate content of a selection of Brussels sprout cultivars at 4 consecutive monthly harvests.

The date of optimum glucosinolate content seems to be correlated with the genetics of the lines. The accumulation of glucosinolates during the development of sprout buttons proceeds until maturity. Overmature sprouts start to reallocate or degrade glucosinolates during the senescence process. On average, the glucosinolate content of Brussels sprouts cultivars is highest in November. Variations in the sum of glucosinolates as a function of plant age and other developmental parameters is also observed in other Brassicas. (McGregor, 1988; Clossais-Besnard and Larher, 1991; Rosa et al., 1994 and 1996).

The glucosinolate content of F1-hybrids at various locations. The glucosinolate content of Brussels sprouts F1-hybrids is relatively stable at different sites an indication that the genotype determines its content for a significant part. Figure 4 shows the relationship between the glucosinolate content of 30 F1-hybrids at location De Schermer and Barendrecht. The glucosinolate content is highly significantly correlated ($r = 0.85$, $p < 0.01$) between the locations. The glucosinolate content in Barendrecht is however significantly lower than in De Schermer (paired two-tail test, $p = 0.001$) indicating that crop production site has an influence on the glucosinolate content of cultivars.

The proportion of sinigrin and progoitrin in the glucosinolate content. The new method effectively can be used to screen Brussels sprout lines and F1-hybrids for glucosinolate content as a marker for a putative high sinigrin and progoitrin content. A high content of the glucosinolates sinigrin and progoitrin is not accepted by consumers and will result in sprouts with a poor taste (Van Doorn et al., 1998a).

In Figure 5 the relationships are presented between the glucosinolate content of cultivars and their sum of sinigrin and progoitrin at respectively locations De Schermer and Barendrecht. The glucosinolate content of cultivars at both locations is for a significant part comprised of sinigrin and progoitrin (De Schermer: $r = 0.79$, $p < 0.01$; Barendrecht: $r = 0.69$, $p < 0.01$). The glucosinolate content of cultivars is in average for 75 and 55 % composed of sinigrin and progoitrin at respectively
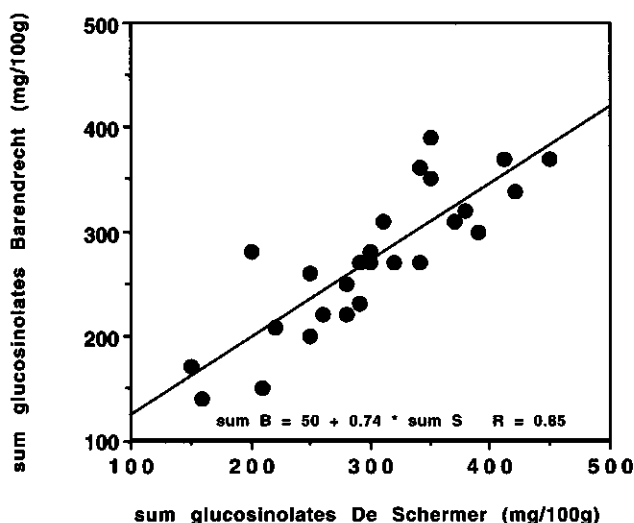
Figure 4. Relationship between the glucosinolate content of 30 F1-hybrids at locations De Schermer (S) and Barendrecht (B).
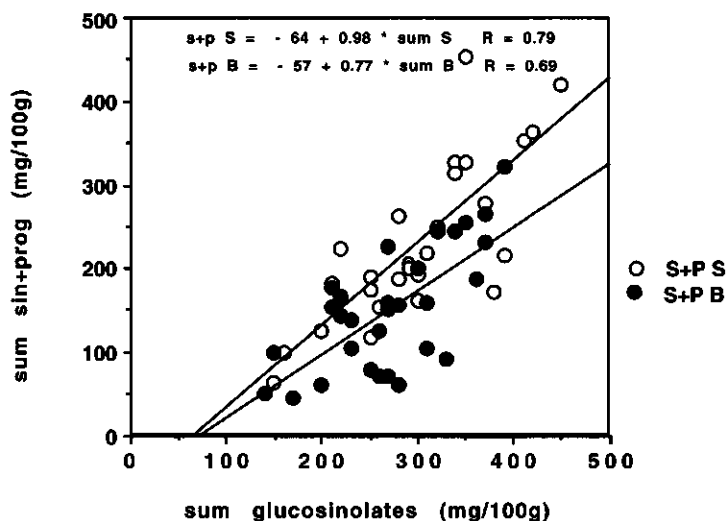
Figure 5. Relationships between the glucosinolate content of 30 F1-hybrids and their sum of sinigrin and progoitrin at the locations De Schermer (S) and Barendrecht (B). The glucosinolate content and the sum of sinigrin and progoitrin are expressed in mg/100g, s+p and sin+prog denote the sum of sinigrin + progoitrin.
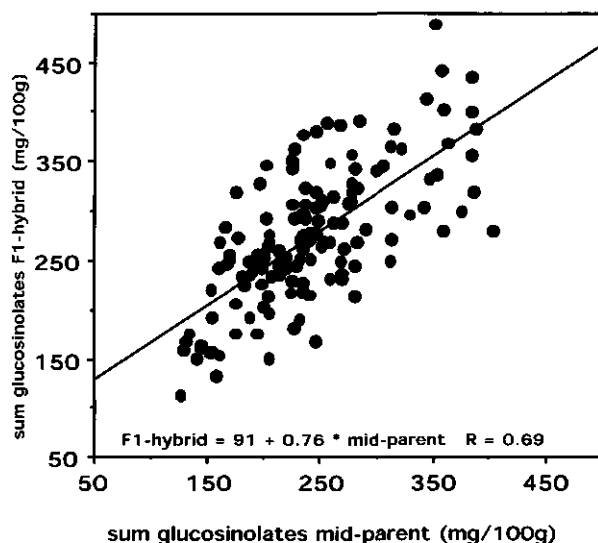
Figure 6. Relationship between the glucosinolate content of the mid-parent (mean content of the corresponding parental lines of a F1-hybrid) and the F1-hybrid.

locations De Schermer and Barendrecht. These results suggest that the glucosinolate determination is a reasonable marker for the sum of these two glucosinolates.

The glucosinolate content of F1-hybrids and corresponding parental lines. The glucosinolate content of F1-hybrids is proportional to the content observed in the corresponding parental lines. Figure 6 shows that the glucosinolate content of midparents and the corresponding F1-hybrids is significantly correlated ($r = 0.69$, $p < 0.001$). In Figure 6, considerable deviations between the mid-parent and F1-hybrid values are observed for many combinations, suggesting that the sum of glucosinolates in the F1-hybrids is only partly related to the sum of glucosinolates of the parental lines.

The new method has proven its accuracy and reliability for the large scale determination of aliphatic glucosinolates such as sinigrin and progoitrin, which are stable during the sample preparation protocol with phosphoric acid (van Doorn et al., 1998b). It was not checked if the method can also be applied for

the determination of indole glucosinolates. This is however doubtful, as these are very labile under extreme conditions.

Acknowledgment

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Chapter 4
The glucosinolates sinigrin and progoitrin are important determinants for taste preference and bitterness of Brussels sprouts.

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Abstract

The glucosinolates, sinigrin and progoitrin are related to the bitterness observed in Brussels sprouts. The role of these glucosinolates in taste preference for Brussels sprouts (*Brassica oleracea* var. *gemmifera*) was studied with two different consumer taste panels and an analytical panel. Four different taste trials were conducted between 1989 and 1995 to study the role of sinigrin and progoitrin on taste preference and bitterness of sprouts. Sinigrin and progoitrin correlated negatively with taste preference of sprouts when their combined content was higher than 2.2 g kg^{-1}. Under these conditions the overall preference for different Brussels sprouts cultivars, in two different trial designs, was accounted for respectively 64 (Novartis Seeds BV) and 86 % (TNO) by the contents of the glucosinolates sinigrin and progoitrin. In two additional trials, in which the combined content of sinigrin and progoitrin of all cultivars was below 2.2 g kg^{-1}, no relationship between taste preference and the content of both glucosinolates was observed. In the latter trials the content of sinigrin and progoitrin was found to be correlated with bitterness ($r^2_{multiple}$ = 0.67 and 0.93 respectively) as assessed by an analytical panel. The results of the taste trials show that both bitterness and taste preference for sprouts are correlated with the content of sinigrin and progoitrin. Taste preference for sprouts is only correlated with sinigrin and progoitrin when their combined content exceeds 2.2 g kg^{-1}, while bitterness is correlated with both glucosinolates for the whole studied content range.

Introduction

Worldwide, edible parts and seeds of plants belonging to the genus *Brassica* have formed an important part of the diet of animals and humans for a long time. They contain glucosinolates, a group of labile thioglucosides, some of which are toxic, others (indole glucosinolates) having anti-carcinogenic properties when metabolised (McDanell *et al* 1988; Stoewsand *et al* 1988). To date, more than 90 different glucosinolates have been identified. These

can be subdivided into 3 groups: aliphatic, aromatic and indole glucosinolates with respect to their rest group (Tookey *et al* 1980; Fenwick *et al* 1983). Glucosinolates are present in all plant parts and are physically separated from the enzyme thioglucoside glucohydrolase (EC 3.2.3.1). This enzyme is able to degrade the glucosinolates to a mixture of the corresponding isothiocyanates, thiocyanates, nitriles, glucose and sulphate. This degradation process starts after disruption of the tissue. Its products are partly responsible for the typical flavour of cruciferous plants.

Brussels sprouts are an important vegetable in the UK, West-Germany, Belgium, France and the Netherlands. Over the last two decades however, complaints have been heard regularly about the taste of some cultivars for the fresh market and the processing industry. It has been shown that the glucosinolates sinigrin and progoitrin are involved in bitterness observed in Brussels sprouts (Fenwick *et al* 1982; Griffiths and Fenwick 1984) and comprise together with gluconapin and glucobrassicin almost the total glucosinolate content in this crop (Heaney and Fenwick 1980a,b; Heaney *et al* 1983). Although bitterness is an important taste attribute, its role in taste preference of consumers for Brussels sprouts has never been studied. Taste preference of sprouts may not only be determined by bitterness but also by other taste attributes such as sweetness. The interplay between the taste attributes seem to determine whether sprouts are sweet, neutral or bitter (Bedford 1989).

The aim of the study is to study the impact of the glucosinolates sinigrin and progoitrin on taste and bitterness of sprouts and to establish critical levels of both glucosinolates which do not result in complaints about bitterness and taste by consumers.

Experimental

Taste assessment of Brussels sprouts by consumer and expert taste panels.

Assessment of the taste of Brussels sprouts when included in a meal.
A consumer taste panel of 48 members, consisting of 24 men and 24 women, assessed 10 Brussels sprouts cultivars for taste on 3 consecutive weeks. The trials were conducted to study the relationship between the content of sinigrin and progoitrin and consumer preference when sprouts were included in a regular meal. On occasion, 4 cultivars were assessed for taste. During the second and third occasion, one cultivar from the previous week was re-assessed. Brussels sprouts were served as a vegetable during a complete meal consisting of a starter, main course with Brussels sprouts, potato variation, meat, wine and a dessert. Each week, the 48 panel members were randomly supplied with the four different cultivars at 10 minute intervals.

The Brussels sprouts were coded with a random three digit number. In this experimental design, panel members on the same table never assessed the same Brussels sprouts cultivars simultaneously. Three buttons were supplied for each cultivar.

Taste was scored on a form with a linear scale of 0-100 mm (0, very bad; 100, excellent). The members of the taste panel were asked to use the full scale to score the taste. They were not allowed to consult their earlier assessments to compare their scoring consistency.

On the first occasion the Brussels sprouts cultivars Rampart, Tardis, Kundry and Ajax were assessed for taste, on the second occasion Icarus, Adonis, Pallas and Rampart and on the last occasion Phylemon, Skios, Lauris and Adonis. Rampart was retested on occasion 2. Adonis was used for this purpose on occasion 3.

Taste trials with professional expert panels and consumer panels.
Between 1991 and 1995, three taste trials have been conducted at the CIVO-TNO institute in Zeist, The Netherlands. The trials were

conducted to confirm the relationship between the sinigrin and progoitrin content of cultivars and consumer preference and to assess critical levels of glucosinolates that are still acceptable to consumers. During each trial eight samples of sprouts were assessed for taste by a 50-member consumer taste panel. All three trials were conducted on two consecutive days. The consumers were seated in individual cabins in an odour-free room with neutral fluorescent tube lighting. During the trials crackers and water were freely available. The sprout samples were coded with a random three-digit number and served randomised to the members of the panel. The first day the sprout samples were tested by half of the panel, the second day the other half of the panel was active. Taste was scored on a category scale: 1 = excellent, 2 = very good, 3 = good, 4 = reasonable, 5 = not satisfactory, 6 = bad, 7 = very bad.

In the first trial, on January 1991, seven cultivars were evaluated for taste. One cultivar was tested twice as a control for reproducibility, making a total of eight samples.

In the second and third trial, on November 1993 and January 1995, sprouts from eight different cultivars were evaluated for taste according to the same experimental design. Both trials were organized by the CBT (Centraal Bureau van de Tuinbouwveilingen) and the PAGV (Proefstation voor Akkerbouw en Groentegewassen in de Vollegrond). During the latter trials the sprout samples also were assessed for bitterness by a 10-member expert panel. The aim of these assessments was to study the relationship between the bitterness of sprout samples and consumer preference. The samples were randomly evaluated 1-3 times for bitterness by the panel on a line scale of 100 units (0, very weak; 100, very strong). The trials were organised similarly to those for the consumers: In individual odour-free cabins equipped with neutral fluorescent tube lighting, crackers and water were freely available, the sprout samples were coded with a random three-digit number and served randomised to the members of the panel.

Brussels sprouts cultivars used in taste trials.

Brussels sprouts for the first taste trial at Novartis Seeds BV were obtained from the cultivar assessment trials of Novartis Seeds BV in Enkhuizen, in the north of the Netherlands. Sprouts

were grown under standard conditions on loam soil. For this trial (at Novartis Seeds BV, Enkhuizen) the cultivars Rampart (cultivar of Royal Sluis, Enkhuizen, The Netherlands), Tardis, Kundry, Lauris, Icarus, Pallas, Adonis , Phylemon, Ajax and Skios (all varieties of Novartis Seeds BV) were used. For the first taste trial at TNO Nutrition and Food Research Zeist, The Netherlands, the cultivars Kundry, Lauris, Edmund, Rampart, Phylemon, Ajax and Adonis were used.

The second and third taste trial at TNO Nutrition and Food Research were conducted with coded cultivars as agreed with the contributing breeding companies. The cultivars were coded as the aim of the trials was to study the relationship between the content of sinigrin and progoitrin of sprout samples, but not cultivars, with bitterness and taste preference of consumers. The sprout samples from the various cultivars were obtained from the PAGV institute in Lelystad, The Netherlands.

Processing of Brussels sprouts.

Brussels sprouts were harvested the day before the taste trials. Brussels sprouts of category A (size: 2-3 cm) were used for the assessment trials. The outer leaves were removed and buttons were dropped into boiling water without salt and cooked for 15 minutes. During the taste trials, the cooked Brussels sprouts were stored in a thermostated storage vessel at 45°C.

Determination of sinigrin and progoitrin.

Glucosinolates were extracted directly from freshly harvested samples of Brussels sprouts (200 g for each cultivar) as described by van Doorn et al. (1998). Representative samples of 200 g of buttons were taken from the samples harvested for the taste trial for the determination of glucosinolates.

The content of the glucosinolates sinigrin and progoitrin was determined with specific immunochemical assays. The sinigrin assay was log linear between 10 nM and 10 μM sinigrin and showed a cross reactivity of 7.4% to gluconapin and a negligible cross reactivity to the glucosinolates progoitrin and glucocheirolin. The progoitrin assay was log linear between 10 nM and 10 μM progoitrin and showed a cross reactivity of 4.6% to the glucosinolates sinigrin, gluconapin and glucocheirolin. The Brussels sprout samples were diluted to bring them into the

linear range of the assay. Contents are expressed in g kg⁻¹ fresh weight. The content of sinigrin and progoitrin of samples as obtained by specific Elisa assays were standardized for HPLC values as described by van Doorn *et al.* (1998).

Results

Taste trials in which sprouts are included in a meal.
Brussels sprouts are exclusively consumed as part of a meal and not seperately. Therefore, a test was designed in which consumer preference for Brussels sprouts could be assessed in a normal meal. The results of this taste trial with 10 Brussels sprouts cultivars are presented in table 1. The cultivars were sorted into three groups of four cultivars according to the day of testing. Taste is expressed as the mean value of all members of the panel. The assessed taste was normally distributed for all cultivars (test for skewness and curtosis). The cultivars Rampart and Adonis were used as references to correct the scores of all cultivars, tested on different evenings, to a comparable level. The preference for Rampart and Adonis was in both cases less in the second assessment. It was clear from comments of the panel members that most of them did not eat Brussels sprouts more then once a week. This probably induced a negative bias, which was reflected in a lower appreciation as the test progressed. To correct for this phenomenon, the mean scores were brought up to those of the second week and expressed as standardized mean in Table 1. Significant differences in taste were observed between the cultivars every week. (paired two tail Student's t-test, $p < 0.02$). The content of sinigrin and progoitrin of the cultivars in the trials varied, respectively, from 1.3-4.9 and 0.7-2.1 g kg⁻¹. The mean standardised taste of cultivars of Brussels sprouts correlated with the content of progoitrin ($r^2 = 0.40$, $p = 0.04$) and sinigrin ($r^2 = 0.49$, $p < 0.01$). Using multiple regression, 64 % ($p=0.01$) of the variation in taste could be accounted for by the glucosinolates sinigrin and progoitrin.

Figure 1 shows the relationship between the sinigrin and progoitrin content of sprout cultivars and consumer taste preference as calculated with multiple regression.

In this trial, progoitrin seems to have a more negative impact on the taste rating than sinigrin.

Taste trials with consumer panels and expert panels in which sprouts are not included in a meal.

The observed relationship between assessed taste by consumers and the glucosinolates sinigrin and progoitrin, as observed in the first trial, was retested three times between 1991 and 1995 at the institute TNO for Nutrition and Food Research. During the first trial, in November 1991, seven sprout cultivars were assessed for taste by a consumer panel of 50 housewives. The average score for taste, relevant remarks on taste and the sinigrin and progoitrin content of the cultivars are presented in Table 2 (trial 1). Significant differences in taste were observed between the cultivars (least significant difference (LSD) = 0.46, p = 0.05). The average taste of the cultivars varied between 3.72 (almost reasonable) and 4.94 (almost not satisfactory).

TABLE 1. The content (g kg^{-1})of sinigrin and progoitrin and the taste rating (mm on 100 mm scale) of 10 Brussels sprouts cultivars during taste trials on three successive occasions at location Novartis Seeds BV [a].

Cultivar	Sinigrin	Progoitrin	Sum	Taste rating (scale 0-100)		
	(g kg^{-1})	(g kg^{-1})	(g kg^{-1})	Mean	St mean[b]	SD[c]
week 1						
Rampart	3.0	0.7	3.7	56	49	3.9
Kundry	2.0	1.6	3.6	46	40	3.6
Ajax	3.9	1.2	5.1	44	39	4.0
Tardis	4.9	2.8	7.7	41	36	3.6
week 2						
Icarus	2.1	0.7	2.8	53	53	3.4
Adonis	1.4	1.3	2.7	52	52	3.4
Rampart	2.0	0.9	2.9	49	49	3.9
Pallas	2.5	1.5	4.0	48	48	3.6
week 3						
Skios	3.0	1.1	4.1	47	53	3.7
Adonis	1.4	1.1	2.5	46	52	3.5
Phylemon	1.3	1.8	3.1	46	52	3.9
Lauris	2.3	2.1	4.4	39	44	3.5

[a] Per week, the cultivars are sorted for ascending order of taste rating. Taste rating: 0 mm, very bad; 100 mm, excellent.
[b] St mean, standardised mean taking week 2 as standard.
[c] SD, standard deviation.

Figure 1. Relationship between sinigrin and progoitrin content (g kg^{-1}) of sprout cultivars from Table 1 and consumer taste preference (0-100 mm scale). The average taste of cultivars is correlated with the sinigrin and progoitrin content according to the following equation: Calculated taste = 60.5-4.17 x progoitrin-2.98 x sinigrin. R^2 = 0.64 denotes the square correlation coefficient of the relationship between both parameters.

The cultivars *Adonis* and *Kundry* had a significantly better taste than the other cultivars. *Edmund* had a significantly worse taste than all other cultivars. The sinigrin and progoitrin content of the cultivars in this trial varied, respectively, from 1.0-4.6 to 0.8-4.1 g kg^{-1}, comparable values with those of the first trial at Novartis Seeds BV. Again, there was a significant correlation between the content of both sinigrin and progoitrin and assessed taste. The partial regression coefficients of the relationships between respectively sinigrin (r^2 = 0.86, $p < 0.01$) and progoitrin (r^2 = 0.71, $p < 0.01$) with taste are highly significant.

Using multiple regression, both glucosinolates accounted for 86% ($p < 0.01$) of the variation in taste. Figure 2 shows the weighed relationship between sinigrin and progoitrin, as calculated with multiple regression analysis, and the average taste as assessed by the panel. Remarks from the panel members for specific

TABLE 2. The content (g kg⁻¹) of progoitrin and sinigrin, the sum of progoitrin and sinigrin, bitterness, consumer taste ratings (and remarks) of Brussels sprouts cultivars during three taste trials[a].

Cultivar code nr	Progoitrin (g kg⁻¹)	Sinigrin (g kg⁻¹)	sum (g kg⁻¹)	Bitterness (0-100)	Taste (1-7)	% consumers with remarks		
						too bitter	poor taste	good taste
Trial 1: January 1991								
Adonis	1.9	1.0	2.9	nd	3.72	2	20	40
Kundry	2.9	1.1	4.0	nd	3.72	10	38	16
Phylemon	4.1	1.3	5.4	nd	4.21	3	24	17
Ajax	1.4	3.8	5.2	nd	4.22	6	32	20
Lauris	3.9	1.3	5.2	nd	4.40	10	38	16
Rampart	0.8	3.8	4.6	nd	4.42	8	26	10
Edmund	3.6	4.6	8.2	nd	4.94	18	56	2
Mean	2.7	2.4	5.1	nd	4.23	8	33	17
					LSD = 0.46			
Trial 2: November 1993								
1	0.6	0.4	1.0	38.6	3.70	2	13	30
2	0.4	0.2	0.6	31.6	3.71	2	20	30
3	0.6	0.4	1.0	41.1	3.79	4	20	27
4	0.7	0.6	1.3	42.0	3.95	4	21	25
5	0.6	1.1	1.7	42.5	4.02	9	18	21
6	0.4	0.7	1.1	29.1	4.05	0	20	30
7	0.5	0.4	0.9	32.2	4.14	5	18	11
8	0.3	1.1	1.4	40.2	4.18	14	32	20
Mean	0.5	0.6	1.1	37.2	3.94	5	20	24
				NS	NS			
Trial 3: January 1995								
1	0.2	0.3	0.5	28.6	3.83	4	14	33
2	0.3	0.2	0.5	35.2	3.93	0	21	63
3	0.9	0.7	1.6	62.0	4.06	28	40	40
4	0.6	1.5	2.1	69.3	4.12	35	30	42
5	0.3	0.6	0.9	45.0	4.19	7	47	21
6	0.6	1.6	2.2	75.4	4.34	37	68	16
7	0.8	0.9	1.7	58.5	4.36	21	49	23
8	0.8	0.6	1.4	63.6	4.51	16	63	5
mean	0.6	0.8	1.5	54.7	4.17	19	42	30
				LSD = 6.5	NS			

[a] The cultivars are specified (trial 1) or coded by number (trials 2 and 3) and sorted for ascending order of consumer taste rating. Taste rating: 1, excellent; 7, very bad. The bitterness rating in trials 2 and 3 is scored on a 0-100 scale (0, very weak; 100, very strong). When applicable, the least significant differences (LSD) at p = 0.05 for the parameters bitterness and taste are given underneath the means. Abbreviations: NS = not significant, ND = not determined.

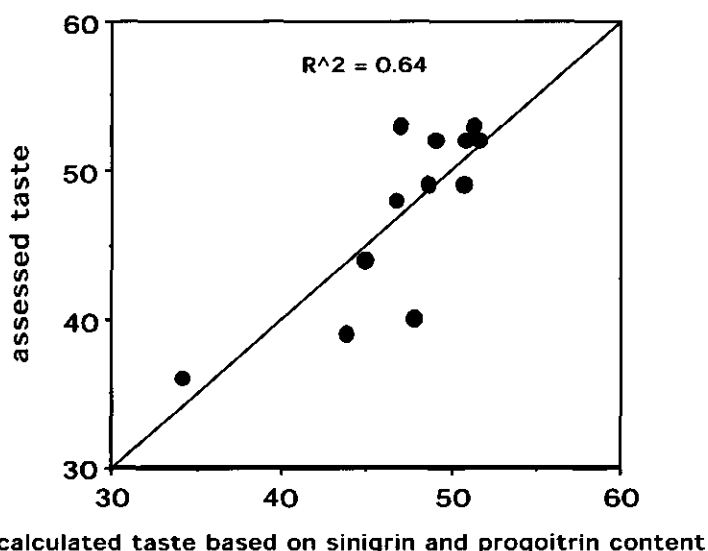calculated taste based on sinigrin and progoitrin content
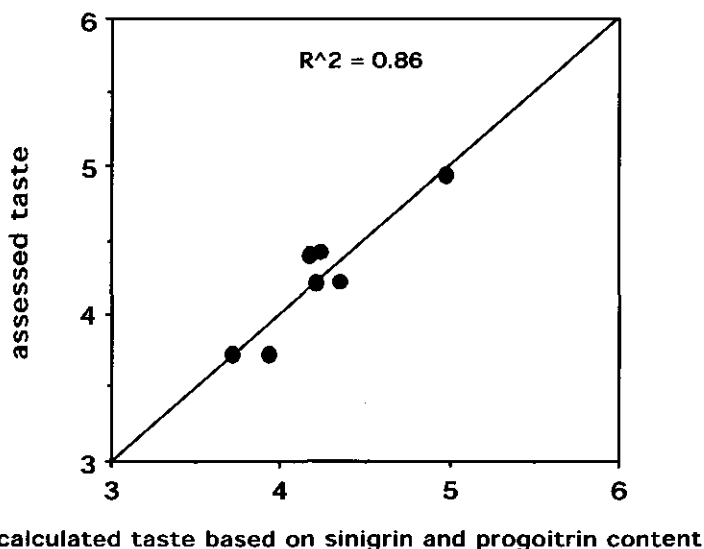
Figure 2. Relationship between sinigrin and progoitrin content (g kg^{-1}) of sprout cultivars from trial 1 of Table 2 and consumer taste preference (1-7 scale). The average taste of cultivars is correlated with the sinigrin and progoitrin content according to the following equation: Calculated taste = 3.09 + 0.19 x progoitrin + 0.26 x sinigrin. R^2 = 0.86 denotes the square correlation coefficient of the relationship between both parameters.

cultivars on aspects such as bitterness, poor taste and good taste are listed in the right columns of Table 2 and should be in agreement with the average taste score for the cultivars. For the first trial, taste is associated with the percentage of consumers giving the remark 'too bitter'. The percentage remarks 'too bitter' is proportional to the percentage remarks 'poor taste'. The remarks on taste aspects demonstrate that the variation in taste is associated with differences in bitterness between cultivars. In the second trial (trial 2 of Table 2) the cultivars did not differ significantly for taste (p = 0.11). The average taste of the cultivars varied between 3.70 and 4.18, all values close to reasonable. The cultivars of the second trial were also assessed for bitterness but did not differ for this aspect (p = 0.13). The sinigrin and progoitrin content of the cultivars was considerably

lower in comparison to the previous trials and varied, respectively, from 0.2 to 1.1 and 0.3 to 0.7 g kg^{-1}. The sum of sinigrin and progoitrin did not exceed the level of 1.7 g kg^{-1}. Such levels apparently are too low to result in significant differences in taste and bitterness between cultivars. On average, remarks of the consumers with respect to 'too bitter' and 'poor taste' are not mentioned often (respectively 5% and 20%), another indication that the assessed cultivars in the second trial combined a reasonable taste with a low level of bitterness. Bitterness of the sprout samples was, despite the relative small differences, correlated with the sinigrin and progoitrin content ($r^2_{multiple}$ = 0.67).
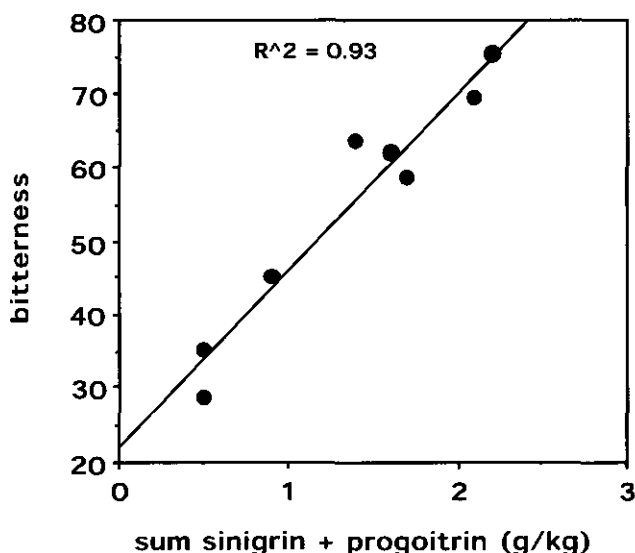
Figure 3. Relationship between bitterness (0-100 scale) of sprout samples and the sum of sinigrin and progoitrin (g kg^{-1}). Bitterness of cultivars is correlated with the sinigrin and progoitrin content according to the following equation: Bitterness = 22.3 + 27.6 x progoitrin + 21.8 x sinigrin. R^2 = 0.93 denotes the square correlation coefficient of the relationship between both parameters.

In the third trial (trial 3, Table 2) again no significant differences in taste were observed between cultivars at the p =

0.05 level. The taste of the cultivars varied between 3.83 and 4.51, values which correspond with a reasonable to almost not satisfactory taste. The cultivars were, however, found to differ significantly for bitterness by the expert panel at a significance level of p = 0.05. In Fig 3 it is shown that bitterness of the cultivars is highly correlated with the content of sinigrin and progoitrin ($r^2_{multiple}$ = 0.93, p < 0.01) with both compounds contributing to the same extent to bitterness. Apparently, the appreciation of consumers for sprout cultivars is unlinked to bitterness at the specified glucosinolate contents of sinigrin and progoitrin. Bitterness of the cultivars, as assessed by the expert panel, is associated with the percentage remarks 'too bitter' from consumers. Figure 4 shows the association between assessed bitterness by an expert panel and the percentage remarks on bitterness by consumers for the cultivars of trial 3 (Table 2).

Figure 4. The association between bitterness of Brussels sprout cultivars and the percentage remarks 'too bitter' by consumers during taste trial 3 (Table 2).

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Discussion

Taste trials with consumer panels and expert panels were conducted to elucidate the role glucosinolates play in bitterness and taste preference of Brussels sprouts. The factors and compounds that determine the taste preference of consumers for Brussels sprouts have not previously been reported. From early work of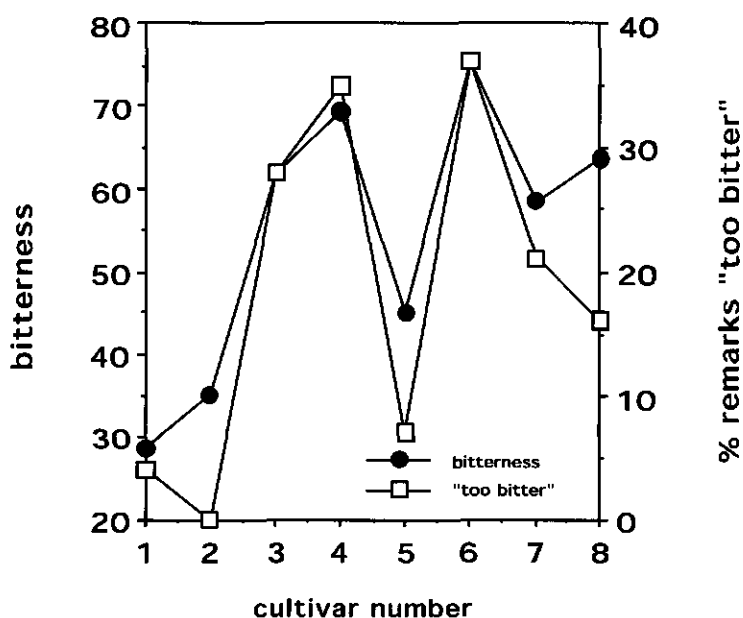 Fenwick *et al* (1983) and Griffiths and Fenwick (1984) we know that the glucosinolates sinigrin and progoitrin are associated with a bitter taste. More recently Bedford (1993) confirmed the role of sinigrin and progoitrin in bitterness of Brussels sprouts. Sinigrin has a bitter taste as 71% of a group of assessors mentioned its bitterness at a concentration of 50 mg.100 ml^{-1} (Fenwick *et al* 1983). Progoitrin is a non-bitter glucosinolate which, however, can be degraded enzymatically by the enzyme thioglucosidase or by heat treatment to the extremely bitter compound goitrin, a compound which can occassion comment by assessors at a concentration of 1.2 mg.100 ml^{-1}. Nothing is known about the maximum level of both glucosinolates in Brussels sprouts which does not result in complaints from consumers with respect to bitterness and taste.

The contribution of progoitrin to taste preference and bitterness, by means of its degradation product goitrin, may vary depending on the processing conditions prior to consumption. In our taste trials, all Brussels sprouts samples were processed in a similar way to exclude unequal degradation of glucosinolates due to differences in processing conditions between samples. Gluconapin also is a bitter glucosinolate but present at such a low level in sprouts that it is expected to have no impact on bitterness (Heaney and Fenwick 1980a, b).

In this study Brussels sprout samples were evaluated for taste and bitterness by consumer panels and expert panels in an integrated approach. The taste of Brussels sprout cultivars varied between almost reasonable (3.70, trial 2) and almost not satisfactory (4.94, trial 1) in the three trials of Table 2, a difference of 1.24 on a 7-point scale. The variation in taste in Table 1, between 39 and 56 on a 100 unit scale, relatively speaking is in line with the taste figures of Table 2. Consumers

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on average do not use the terms 'good' and 'bad' for sprout cultivars which vary from 0.5 to 8.2 g kg^{-1} in combined sinigrin and progoitrin content.

Figure 5 shows that the pooled individual scores for cultivars in the three taste trials are normally distributed around the term reasonable. Consumers apparently use the middle part of the scale to give their score for taste.

Sinigrin and progoitrin were found to have an impact on consumer taste preference for Brussels sprout samples. In the first trial, in which sprouts were served as part of a meal, it was clearly demonstrated that taste preference of consumers for sprouts was correlated with the content of sinigrin and progoitrin ($r^2_{multiple} = 0.64$). The relationship between the content of sinigrin and progoitrin and taste preference was confirmed by the first trial with a taste panel in which sprouts were presented without a meal (Table 2, trial 1, $r^2_{multiple} = 0.86$). The relationship between the sinigrin and progoitrin content and consumer preference is apparantly not dependent on whether the sprouts are consumed in a simple experimental design or as part of a meal.
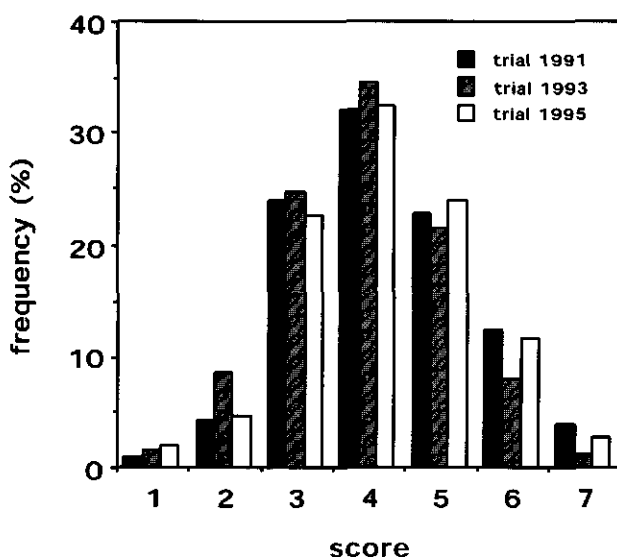

Figure 5. The frequency distribution of individual scores for the cultivars as included in the consumer trials of Table 2.

The scores correspond with the terms in the Materials and Methods section.

Both glucosinolates seem to have a similar negative influence on taste preference of consumers when present above specific levels. The correlations between the content of both glucosinolates and taste were observed with populations of sprout samples in which the sum of sinigrin and progoitrin respectively varied from 2.7 to 7.7 g kg^{-1} (Table 1) and 2.9 to 8.2 g kg^{-1} (Table 2, trial 1). No relationship was observed between the taste of sprout samples and the content of sinigrin and progoitrin when the sum of sinigrin and progoitrin was equal or below 2.2 g kg^{-1} as in the last two trials (trials 2 and 3, Table 2). Apparently a sum of sinigrin and progoitrin equal to 2.2 g kg^{-1} is close to the acceptable content which no longer has a negative impact on taste. A consumer trial in which the sprout samples have a variable sum of sinigrin and progoitrin between 1.0 and 3.0 g kg^{-1} will provide definitive proof that the critical content of the sum of both glucosinolates is close to 2.2 g kg^{-1}. Trials 2 and 3 were two attempts to define the critical glucosinolate level for consumers but on both occasions the glucosinolate content of the cultivars, which normally have a sum of sinigrin and progoitrin of about 3.0 g kg^{-1}, was far lower due to environmental conditions that favour a low sum of both glucosinolates. The combined results of the 3 trials of Table 2 clearly demonstrate that the critical sum of sinigrin and progoitrin must be close to 2.2 g kg^{-1}.

Despite the fact that the sum of sinigrin and progoitrin is not related to taste preference below a content of 2.2 g kg^{-1}, bitterness of the sprout samples is clearly correlated with both glucosinolates at lower contents. In trials 2 and 3 of Table 2, bitterness is correlated with the content of sinigrin and progoitrin (see Fig 3). The consumer will expect and appreciate some bitterness in sprouts. Too much bitterness is not preferred. Our trials combined with the results of Fenwick et al (1983), Griffiths and Fenwick (1984) and Bedford (1993) confirm the role sinigrin and progoitrin play in bitterness of Brussels sprouts.

The relationships between taste attributes and (bio)chemical compounds in vegetables are mostly studied with trained expert panels. Our taste trials show that both consumer panels and expert panels have a consistent opinion with respect to the taste

determining compounds (such as glucosinolates) in vegetables. Our consumer panels were asked to evaluate the overall taste of Brussels sprouts, not the bitterness. The remarks of consumers on bitter taste, however, did correlate well with the observed bitterness by the expert panel.

The bitterness values and the number of remarks 'too bitter' and 'poor taste' differ significantly between trials 2 and 3 of Table 2 while the glucosinolate content of the samples in both trials is in the same range. These results suggest that bitterness of sprouts is, apart from the sum of sinigrin and progoitrin, determined by additional factors. Trial 2 of Table 2 was conducted in November 1993, trial 3 in January 1995, about 8 weeks later in the season. During the latter trial many more remarks 'too bitter' and 'poor taste' were recorded compared to the former trial. The average taste, bitterness (when assessed), and the percentage of negative remarks on taste ('too bitter' and 'poor taste') were much higher in the January and February trials (trials 1 and 3, Table 2) in comparison to the November trial (trial 2, Table 2). In the January 1991 trial, most remarks relate to a poor taste, in the February 1995 trial more remarks are recorded with respect to bitterness. This shift in remarks is probably due to the training of the panel for taste aspects of Brussels sprouts. All three trials were conducted at the same institute with a panel which was for a significant part composed of the same consumers. The consumers may have developed the skill to assess bitterness of sprouts more accurately during the successive trials.

The physiological age of sprouts at the point of harvest might also have an impact on their bitterness and taste. During the season, the glucosinolate content of sprout cultivars is found to increase up to December and afterwards decreases in time. If this decrease of the glucosinolate content in time is due to degradation by the enzyme thioglucoside glucohydrolase, this might be the explanation why the sprouts in the January and February trials have a less acceptable taste and a higher bitterness in comparison to sprouts from the November trial because of, for example, goitrin synthesis from progoitrin. Sprout

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samples with a low sinigrin and progoitrin content will, however, always be less sensitive to bitterness enhancing conditions.

Bitterness of sprouts seem to have no impact on taste preference of consumers below a combined sinigrin and progoitrin content of 2.2 g kg⁻¹. An interesting question is what compounds determine the taste preference of consumers for sprouts when glucosinolates do not effect taste anymore?. Our data suggest that important taste determining factors are lacking as no significant differences between cultivars are observed below the 2.2 g kg⁻¹ limit. Bedford (1993) considers sweetness, amongst other taste attributes, to be important for the taste of sprouts in one of the agrifood reports of the Campden Food Preservation Research Association (CFDRA) in the UK. The National Institute of Agricultural Botany (NIAB) in the UK is using, apart from bitterness, the taste attributes sweetness and pleasantness to describe the flavour of cultivars. The annual reports from both institutes suggest that sweetness is an important positive taste attribute in sprouts. In our trials sweetness is not mentioned as a relevant taste attribute as no remarks on sweetness were recorded in any trial. Cultivars with a low bitterness were not appreciated as being sweet. The question arises whether sweetness of sprouts is synonymous with non-bitterness and not a measure for the typical sweet taste which is present many fruits. In the mentioned reports (between 1983 and 1993) of the CFDRA, bitterness and sweetness were always significantly inversely correlated parameters (n = 12 trials, $r^2 = 0.51$-0.88, $p < 0.05$) on the condition that both attributes were significantly different between cultivars. In the various annual NIAB reports, regarding the taste of Brussels sprout cultivars, bitterness and sweetness also are highly significantly inversely correlated (eg $r^2 = 0.55$, $p < 0.01$ in 1992-1993) confirming the CFDRA results. In the mentioned NIAB report pleasantness of cultivars is mainly determined by their bitterness and only for a small part by their sweetness. These observations and our results support the idea that the variation in taste of sprout cultivars only seem to be determined by differences in bitterness. Sprout cultivars contain a sugar content between 3 and 6 g.100g⁻¹ fresh weight (data not shown), which is insufficient to give a sweet sensation in a

glucosinolate-rich background such as sprouts. Sinigrin and goitrin, the degradation product of progoitrin, are already mentioned by consumers at a concentration of respectively 10.6 and 1.2 mg.100 ml⁻¹ (Fenwick *et al* 1983), concentrations which almost always are present in most sprout cultivars and would overrule any sweetness present in sprouts.

The sinigrin and progoitrin content of the six cultivars, which were evaluated for taste in the trial of Table 1 and trial 1 of Table 2, was different between the two trials. The lack of correlation might be due to differences in maturity of the Brussels sprouts at the point of harvest or environmental factors. The Brussels sprout samples used in the taste trials should therefore be regarded as packages of taste and were only used to study the relationship between the glucosinolate content of Brussels sprouts and taste preference by consumers and not for the assessment of the taste of cultivars.

Acknowledgements

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Chapter 5
Quantitative inheritance of the progoitrin and sinigrin content in Brussels sprouts.

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Summary

The quantitative inheritance of the sinigrin and progoitrin content of Brussels sprouts was studied at three production locations in the Netherlands. The content of sinigrin and progoitrin of 30 F1-hybrids varied between the production locations, though their ranking with respect to the content of sinigrin and progoitrin was relatively stable. Using variance analysis the broad sense heritability (i.e. the genetic contribution to total variance) was found to be 0.79 and 0.77 for sinigrin and progoitrin respectively, an indication of a high genetic component in the content of both glucosinolates in F1-hybrids.

The content of sinigrin and progoitrin inherits as a proportion of the average content of the corresponding parental lines. For sinigrin and progoitrin narrow sense heritability values of respectively 0.72 and 1.09 were calculated using 150 F1-hybrids and their corresponding parental lines. The high narrow sense heritability values obtained confirm that breeding for a low content of both glucosinolates in Brussels sprouts is possible.

Introduction

The glucosinolates sinigrin and progoitrin are responsible for the bitterness observed in Brussels sprouts (Fenwick et al., 1983, Griffiths & Fenwick, 1984). Along with gluconapin and glucobrassicin they are the main glucosinolates in Brussels sprout cultivars (Heaney & Fenwick, 1980a and b; Heaney et al., 1983). Both glucosinolates were found to have a negative influence on the preference of consumers for Brussels sprouts (Van Doorn et al., 1998a). In two independent consumer taste trials respectively 63 and 81% of the variation in overall preference could be explained by the content of sinigrin and progoitrin with both glucosinolates having a comparably negative role.

The relationship between a high content of sinigrin and progoitrin and poor flavour of Brussels sprouts suggests that the flavour of sprouts would be improved by decreasing the content of both these glucosinolates. However, a breeding program to

improve flavour will only be efficient when -1- the heritability for the content of sinigrin and progoitrin is sufficiently high to allow rapid progress, -2- only a few key genes determine their content -3- selection for a low content does not have a negative effect on other important agronomic traits and -4- the content of both glucosinolates can be measured reliably on a large scale.

The heritability of a complex trait such as sinigrin or progoitrin biosynthesis is controlled by multiple genes and environmental factors. Variation in the absolute content of specific glucosinolates in cultivars seem to be predominantly determined by environmental conditions. In *Brassica napus* the seed glucosinolate content of low glucosinolate containing cultivars is influenced by environment (Josefsson & Appelqvist, 1968) and nutrition (Josefsson, 1970). Freeman & Mossadeghi (1972) found high significant correlation coefficients between the amount of glucosinolate derived volatiles from various edible plant parts and the sulphate content of the soil the plants were grown on. Neil & Bible (1973) found the glucosinolate content of radish roots to be higher on organic soil than on loam soil. According to Heaney et al. (1983), the content of the glucosinolates sinigrin, progoitrin and gluconapin decreases with increased application of nitrogen fertilizer (0-250 kg/hectare).

The glucosinolate content of Brassicas is also affected by physiological factors. The glucosinolate content of plants is affected by parameters such as leaf age and position on the plant (Pocock et al., 1987; Porter et al., 1991), mechanical wounding (Koritsas et al., 1991), infestation by various insects (Birch et al. 1992), and infection by pathogens (Ludwig-Muller et al., 1996). Jasmonates and salicylic acid stimulate the accumulation of indole glucosinolates in various Brassicas (Kiddle et al., 1994; Bodnaryk, 1994; Doughty et al., 1995). Verkerk et al. (1997) observed an up to 15-fold increase of indole glucosinolates in chopped white cabbage.

Different species of Brassica vegetables have their own characteristic glucosinolate patterns (VanEtten et al., 1976 and 1980; Daxenbichler et al., 1979; Carlson et al., 1981; Sones et al., 1984; Hill et al., 1987; Lewis et al., 1987 and 1988). The content of specific glucosinolates generally matched the mean value of

both parents (Heaney et al., 1983). These are indications that the relative content of specific glucosinolates in varieties of cruciferous vegetables is determined by their genetic background.

Many of the enzymes and/or genes which are involved in the biosynthesis of glucosinolates are well characterized. The biosynthesis routes for aromatic glucosinolates, such as indole glucosinolates, and aliphatic glucosinolates are known to act independently (McDanell et al., 1988). Sinigrin and progoitrin are, along with most other aliphatic glucosinolates, synthesized from the amino acid methionine. Tryptophane is the precursor of all indole glucosinolates. The biosynthesis route of aliphatic glucosinolates such as sinigrin and progoitrin is comprised of side chain extension steps (Magrath et al., 1994; Glover et al., 1988; Chapple et al., 1990), the conversion of extended amino acids into N-hydroxyamino acids (Bennett et al., 1995), the conversion of N-hydroxyamino acids via aldoximes into thiohydroximates (Rossiter et al., 1990), the attachment of glucose moieties to thiohydroximates (Jain et al., 1989) and, finally, the attachment of sulphate moieties to complete the glucosinolates (Glendening & Poulton, 1988; Jain et al., 1989 and 1990). The biosynthesis route described yields glucosinolates with a variable chain length and a terminal thiomethyl-group. The side-chains of these glucosinolates can enzymatically be further modified to yield alk(en)yl-glucosinolates and hydroxy-alk(en)yl glucosinolates (Mithen et al., 1995). The biosynthesis of sinigrin and progoitrin from methionine will take 7 and 9 steps respectively, and is under multigene control. The complexity of the biosynthesis routes suggests that breeding for a low content of both glucosinolates in Brussels sprouts could be time consuming.

In this paper the role of the genotype and environmental variation on the content of sinigrin and progoitrin in Brussels sprouts will be presented using a quantitative approach. Breeding for a low glucosinolate content without emphasis on a low sinigrin and progoitrin content might lead to cultivars with a low indole-glucosinolate content. As indole glucosinolates are believed to play a positive role in the prevention of cancer (e.g. Stoewsand et al., 1988) and the biosynthesis of indole

glucosinolates and aliphatic glucosinolates in Brassicas is not linked (McDanell et al., 1988), it was decided to breed for a low sinigrin and progoitrin content. Specific ELISA assays for sinigrin and progoitrin (Van Doorn et al., 1998b) were used to study the inheritance of these glucosinolates in Brussels sprouts. The aim of the study is to develop new, agronomically acceptable sprout cultivars with a low sinigrin and progoitrin content efficiently. The inheritance of these glucosinolates was studied in terms of broad sense and narrow sense heritability. A model of the biosynthesis route of methionine-derived aliphatic glucosinolates, based on the results of this paper, will be presented.

Materials and methods

The sinigrin and progoitrin content of F1-hybrids at various locations: Evaluation of the broad sense heritability of both glucosinolates
Sprouts were obtained from the assessment trials executed by Novartis Seeds BV for commercial F1-hybrids at three different locations in the Netherlands. 30 F1-hybrids were grown under standard conditions in three different trial fields in respectively De Schermer (North Netherlands, loam soil), Groningen (Northeast Netherlands, sandy soil) and in Barendrecht (Midwest Netherlands, loam soil). Mixed 500 g sprout samples of each cultivar were harvested simultaneously at the three locations in the period October to November. Sprouts from the various cultivars were harvested at their optimal harvest time for consumption quality, the period when sprout buttons combine maximum weight with a fresh, green appearance. In general, the optimal harvest time lasts two to three weeks and is dependent on factors such as genetic earliness of the cultivars, cultivation conditions, yield and standing-ability in the field. The content of specific glucosinolates is highest in sprout cultivars at the optimal harvest time (Van Doorn et al., 1998c). Glucosinolates were extracted from fresh sprout samples within one day after picking according to the method of van Doorn et al. (1998b). Sinigrin and

progoitrin containing samples were stored at -20 °C prior to analysis.

The sinigrin and progoitrin content of F1-hybrids and corresponding parents: Evaluation of the narrow sense heritability of both glucosinolates

In 1989, 150 experimental F1-hybrids and their approximately 50 corresponding parent lines were grown in the Novartis Seeds BV trial field in De Schermer, in the North of the Netherlands, on loam soil. The parental lines were chosen for maximum variation with respect to the sinigrin and progoitrin contents. The parental lines showed stable expression of agronomically important traits as desired for good field performance. The F1-hybrids and parent lines were grown under standard conditions and sprouts of both the F1-hybrids and their corresponding parental lines were harvested at their optimal harvest time for consumption quality. The sinigrin and progoitrin content of the buttons was determined immediately after harvest.

Determination of glucosinolates

The sinigrin and progoitrin content of sprout samples was determined with specific ELISA assays as described by Van Doorn et al. (1998b). Parental lines and F1-hybrids of the heritability study were characterized for the glucosinolate distribution by reversed-phase high performance liquid chromatography (HPLC) according to Minchinton et al. (1982).

Genetic and environmental aspects that determine the inheritance of glucosinolates: Statistical evaluation

The inheritance of specific glucosinolates can, after Suzuki et al. (1989), be specified in terms of broad sense - and narrow sense heritability. The genetic and environmental variance in glucosinolate content, parameters which are necessary for the estimation of the broad sense heritability of the glucosinolate content, were calculated from the results of the F1-hybrid field trials at three different locations (as specified in this section) using factorial ANOVA analysis. Broad sense heritability (H^2) is defined as genetic variance (s^2_g) divided by phenotypic total variance (s^2_p). The environmental variance (s^2_e) is defined as phenotypic variance minus genetic variance.

The narrow sense heritability (h^2) of the glucosinolate content is defined as the slope of the relationship between the glucosinolate content of the mid parent (the average glucosinolate content of parent 1 and 2) and the F1-hybrid. The relationships, and relevant slopes, were calculated with linear regression analysis using the results from the field trial with F1-hybrids and corresponding parental lines, as described in this section.

Results

The sinigrin and progoitrin content of F1-hybrids at different locations
The influence of location on the progoitrin and sinigrin content of Brussels sprouts cultivars was studied at three different sites in the Netherlands in 1989. F1-hybrids of Brussels sprouts showed considerable variation in sinigrin and progoitrin content between sites.

Table 1 shows the sinigrin and progoitrin content of the 30 cultivars at the three different locations. At the bottom of Table 1 the parameters are summarized in terms of average value, standard deviation and range. The cultivars differed significantly in the content of sinigrin and progoitrin (factorial ANOVA, p=0.0001, LSD sinigrin = 0.50 g.kg^{-1}, LSD progoitrin = 0.66 g.kg^{-1}). The content of sinigrin and progoitrin of cultivars was significantly higher at De Schermer compared to Groningen and Barendrecht (factorial ANOVA, p=0.02 and LSD = 0.27 for sinigrin, p=0.005 and LSD = 0.34 for progoitrin). The content of sinigrin and progoitrin of cultivars were not correlated at any location. Except for location De Schermer, the sinigrin content was on average significantly higher than the progoitrin content (paired two-tail t-test: De Schermer = not significant; Groningen, p = 0.007; Barendrecht, p = 0.03).

Figure 1 shows the relationships between the sinigrin content of the cultivars grown in Groningen, Barendrecht and De Schermer. The sinigrin content of cultivars in Groningen was highly significantly correlated with the content at Barendrecht (p<0.01, r^2 = 0.67) and De Schermer (p<0.01, r^2 = 0.59) and between

Barendrecht and De Schermer ($p < 0.01$, $r^2 = 0.67$). Figure 2 shows the relationships between the progoitrin content of these cultivars at the three locations. Also, the progoitrin content correlates highly (r^2 values of respectively 0.82, 0.70 and 0.87, $p < 0.01$) between the locations studied. In Groningen and Barendrecht the cultivars had a comparable progoitrin content while they contained approximately 50% more progoitrin in De Schermer, as indicated by the slopes of the lines.

Table 1. The content of sinigrin and progoitrin in 30 F1-hybrids as grown on three sites in The Netherlands.

cultivar	Schermer		Groningen		Barendrecht	
	sinigrin ($g \ kg^{-1}$)	progoitrin ($g \ kg^{-1}$)	sinigrin ($g \ kg^{-1}$)	progoitrin ($g \ kg^{-1}$)	sinigrin ($g \ kg^{-1}$)	progoitrin ($g \ kg^{-1}$)
1	1.2	0.3	0.6	0.2	0.8	0.2
2	1.8	0.5	0.8	0.3	1.2	0.3
3	1.8	1.2	1.6	1.1	1.3	0.9
4	2.1	2.0	1.3	0.7	1.7	1.3
5	1.6	0.6	1.2	0.5	1.8	0.4
6	0.7	0.5	0.6	0.3	0.7	0.2
7	1.6	0.7	2.0	0.8	2.0	1.1
8	1.8	1.1	1.6	1.5	1.6	1.1
9	1.3	0.9	1.1	0.8	1.2	0.9
10	1.2	1.8	1.0	1.4	0.9	1.5
11	2.4	0.8	1.8	0.6	1.6	0.6
12	1.4	0.5	1.2	0.6	1.1	0.5
13	0.9	1.0	1.0	0.5	0.7	0.5
14	1.5	1.3	1.3	0.6	1.2	0.8
15	1.3	1.4	0.8	0.7	0.6	0.7
16	1.1	0.8	0.5	0.5	0.5	0.8
17	1.7	0.5	2.3	0.4	1.6	0.4
18	3.2	1.1	2.7	0.7	2.2	0.9
19	1.3	1.5	1.0	0.6	0.5	0.8
20	1.2	1.5	1.0	0.6	0.6	0.9
21	2.1	1.1	1.7	0.6	1.5	0.7
22	2.4	3.0	2.1	2.2	1.7	2.0
23	0.8	0.9	1.0	1.0	0.7	0.9
24	1.7	2.7	1.0	1.5	1.0	1.8
25	2.5	3.5	1.5	2.0	2.1	2.2
26	1.7	2.8	1.5	1.6	1.7	1.8
27	1.4	1.7	1.0	0.9	0.8	0.9
28	1.7	3.1	1.3	1.4	1.2	2.1
29	1.0	1.7	1.0	1.1	0.9	1.0
30	1.9	1.8	1.7	0.8	1.4	0.8
mean	1.6	1.4	1.3	0.9	1.2	1.0
sd	0.56	0.87	0.51	0.49	0.49	0.55
lowest value	0.8	0.3	0.5	0.2	0.5	0.2
highest value	3.2	3.5	2.7	2.2	2.2	2.2

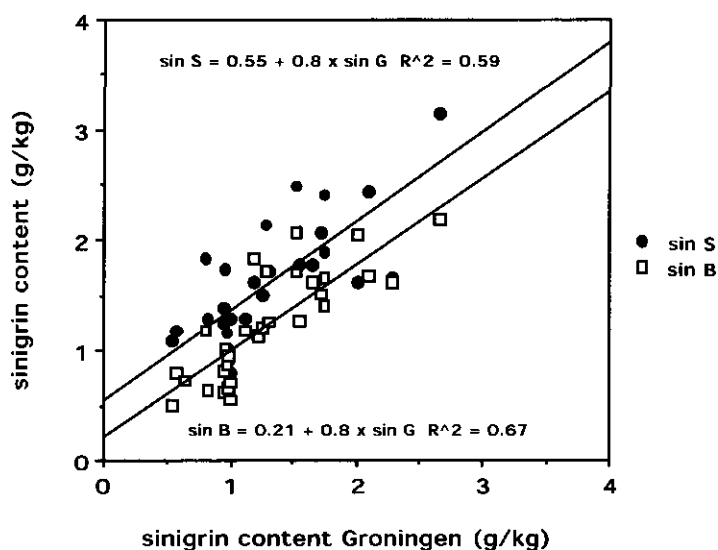*Figure 1.* The relationship between the sinigrin content (g.kg^{-1}) of cultivars grown on sandy soil in Groningen and loam soil in Barendrecht (sin B) and De Schermer (sin S) in the Netherlands.
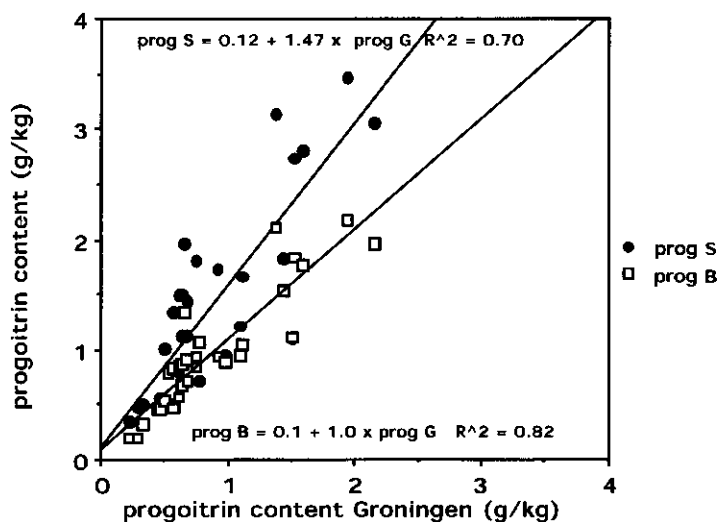

Figure 2. The relationship between the progoitrin content (g.kg^{-1}) of cultivars grown on sandy soil in Groningen and loam soil in Barendrecht (prog B) and De Schermer (prog S) in the Netherlands.

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The broad sense heritability (H^2), genetic variance s^2_g and environmental variance s^2_e in the sinigrin and progoitrin content of Brussels sprouts were calculated using analysis of variance (factorial ANOVA) and the data of the 30 Brussels sprout F1-hybrids at various locations from Table 1. High broad sense heritability values of 0.79 and 0.77 were observed for respectively sinigrin and progoitrin, an indication that cultivars maintain a reasonably constant glucosinolate composition in terms of relative sinigrin and progoitrin content irrespective of environment.

The sinigrin and progoitrin content of F1-hybrids and their corresponding parental lines
The broad sense heritability for a specific trait equals the variation within the phenotype which can be attributed to the variation in the genotype. Whether selective breeding for a trait will be successful is dependent on the narrow sense heritability h^2. The h^2 of quantitative traits such as the content of sinigrin or progoitrin can be calculated from the relationship between the average content in the parents and the content of the F1-hybrid using a large number of parent-F1-hybrid combinations.

The narrow sense heritability of sinigrin and progoitrin was studied in 150 F1-hybrids and their corresponding parental lines. In Figure 3, the typical relationships between the mid parent value and the F1-hybrid value are presented for respectively the content of sinigrin (upper graph) and progoitrin (lower graph). In both graphs the narrow sense heritability is determined by the slope of the equation between the mid parents and F1-hybrids. For sinigrin, a narrow sense heritability of 0.72 is observed. The progoitrin content of the F1-hybrid is somewhat higher than the value of the mid parent, which is reflected in a higher narrow sense heritability for progoitrin (1.09).

Variation in sinigrin and progoitrin content in parental lines and F1-hybrids
The sinigrin content of F1-hybrids (Table 1 and Figure 3), varies from almost 0 (in Figure 3, upper graph) to 3.2 g.kg^{-1} (cultivar 18, location De Schermer, Table 1). The sinigrin content of specific parental lines was found to be as high as 5.0 g.kg^{-1} (data not shown)
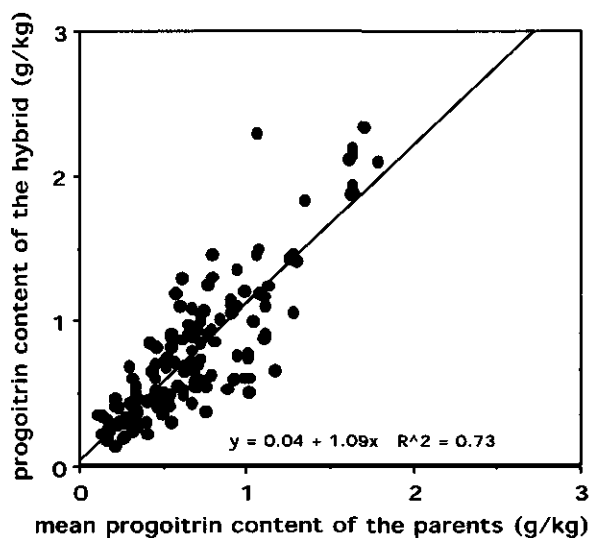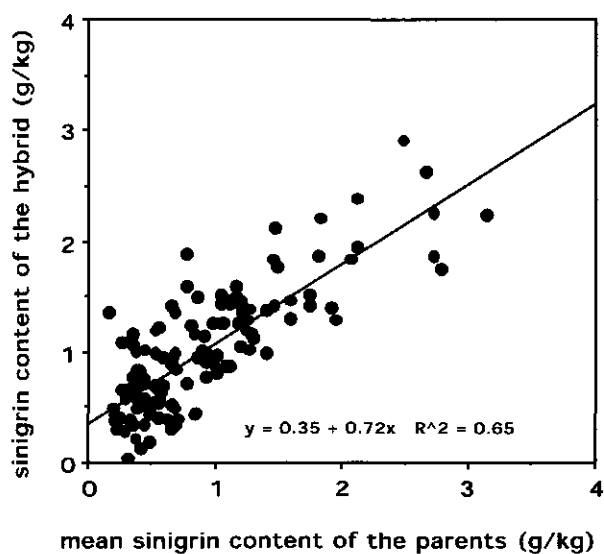
Figure 3. The relationships between the sinigrin (upper graph) and progoitrin (lower graph) content (g.kg^{-1}) of F1-hybrids of Brussels sprouts and the mean content of these glucosinolates in their corresponding parent lines.

The progoitrin content of F1-hybrids (Table 1 and Figure 3, lower graph), varied between 0.2 g.kg^{-1} (cultivar 6, location Barendrecht, Table 1) and 3.5 g.kg^{-1} (cultivar 25, location De Schermer, Table 1). The highest progoitrin content observed in the parental lines was 2.2 g.kg^{-1}, a significantly lower value than the 3.5 g.kg^{-1} which was observed in F1-hybrids constructed with these lines (data not shown). The high progoitrin and sinigrin values of parental lines mentioned are not shown, as they have been used in Figure 3, in which they are plotted as the midparent values on the horizontal axis.

Breeding for optimal levels of sinigrin and progoitrin in Brussels sprouts

Brussels sprouts F1-hybrids will have an optimal flavour when the content of both sinigrin and progoitrin is at an acceptably low level for consumers (Van Doorn et al., 1998a), so parental lines with a low sinigrin and progoitrin content will produce F1-hybrids with a good flavour. Figure 4 shows the sinigrin (upper graph) and progoitrin (lower graph) content respectively of 2 inbred lines which were selected for a low content of these glucosinolates through 5 inbred generations. The selection for a low content was started in the third inbred (F4) generation after 3 inbred cycles with emphasis on an optimal phenotype for agronomic traits such as plant shape, tolerance to pathogens and yield.

The sinigrin content of both lines was still segregating in the F4 as can be seen by the shift for both lines to a low content from the F4 to the F5. In the further generations the sinigrin content of both lines was fixed at a low level. Apparently it is possible to decrease the sinigrin content in specific lines which have already been fixed for their progoitrin content (see line 1 in Figure 4). Figure 4 clearly shows that one of the two lines was already fixed for the content of progoitrin in the F4 generation. In the other line the progoitrin content became constant in the F5 generation. Line 2 demonstrates that a simultaneous decrease in sinigrin and progoitrin content is also possible during inbred generations.
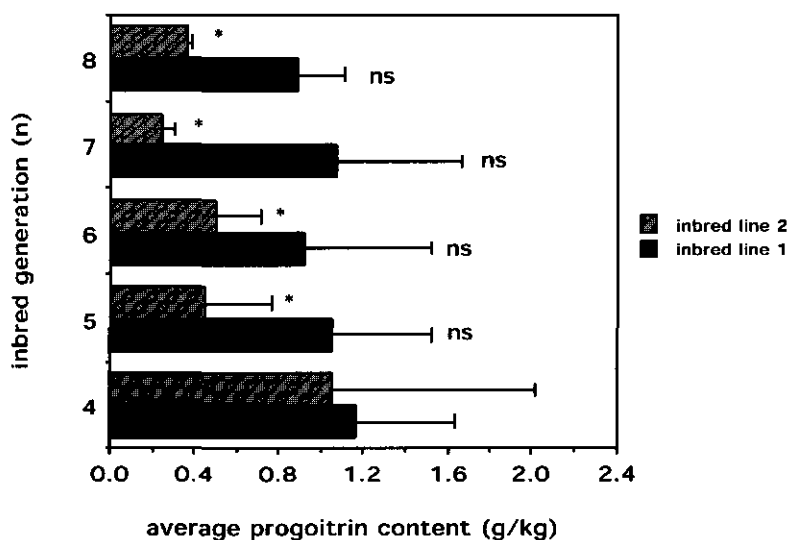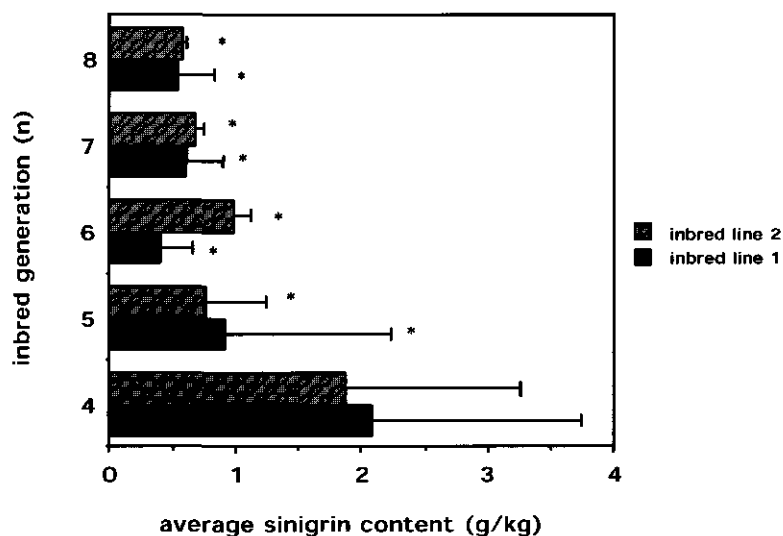
Figure 4. The average sinigrin (upper graph) and progoitrin (lower graph) content (g.kg^{-1}) in plants of the parental lines during the subsequent inbred generations with selection pressure for a low sinigrin and progoitrin content respectively. The horizontal lines denote the standard deviation of the sinigrin or progoitrin content in each generation. ns = not significantly different from generation 4, * = significantly different from generation 4 at p=0.05.

Other glucosinolates in Brussels sprouts parental lines and cultivars.

The parental lines and F1-hybrids used for the heritability studies were, apart from sinigrin and progoitrin, also evaluated for the profile of other aliphatic glucosinolates by means of HPLC. This data was used to screen the germplasm of Brussels sprouts for homogeneous clusters of cultivars and parental lines with particular aliphatic glucosinolate profiles and to screen for possible inter-relationships between the sinigrin and progoitrin content. Such screenings might provide information regarding the design of the biosynthesis route of methionine derived glucosinolates in Brussels sprouts or lead to the identification of critical steps which influence their distribution. The cultivars and lines from our Brussels sprouts breeding program can essentially be divided in three groups with respect to the glucosinolate profile. Table 2 shows the three groups and the corresponding glucosinolates in the groups which have been determined in Brussels sprouts. The glucosinolates were separated and identified by their retention time according to Minchinton et al. (1982). Group 1 is comprised of lines/cultivars that contain glucosinolates which are derived from L-homomethionine. Group 2 is comprised of cultivars containing glucosinolates that are derived from L-homomethionine and L-dihomomethionine (2-amino-6-thiocaproic acid). Group 3 contains a mixture of glucosinolates which are derived from L-homomethionine, L-dihomomethionine (2-amino-6-thiocaproic acid) and L-trihomomethionine (2-amino-7-methylthioheptanoic acid). A highly significant correlation was observed in Brussels sprouts between the gluconapin (butenyl glucosinolate) and progoitrin content (data not shown). The parental lines in the various clusters have been used for the production of the F1-hybrids in Table 1 and Figures 1 to 3.

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Table 2. The glucosinolate profile of the three main groups of cultivars/lines that can be recognized in a breeding program of Brussels sprouts. The glucosinolates and their identity were determined according to Minchinton et al. (1982).

glucosinolate	side chain	trivial name	presence in group		
			1	2	3
allylglucosinolate	$CH_2=CH-CH_2-$	sinigrin	●	●	●
methylthiopropylglucosinolate	$CH_3-S-CH_2-CH_2-CH_2-$	glucoibervirin	●	●	●
3-methylsulfinylpropylglucosinolate	$CH_3-SO-CH_2-CH_2-CH_2-$	glucoiberin	●	●	●
4-methylthiobutylglucosinolate	$CH_3-S-CH_2-CH_2-CH_2-CH_2-$	glucoerucin		●	●
but-3-enylglucosinolate	$CH_2=CH-CH_2-CH_2-$	gluconapin		●	●
2-hydroxybut-3-enylglucosinolate	$CH_2=CH-CHOH-CH_2-$	progoitrin		●	●
pent-4-enylglucosinolate	$CH_2=CH-CH_2-CH_2-CH_2-$	glucobrassicanapin			●
2-hydroxypent-2-enylglucosinolate	$CH_2=CH-CH_2-CHOH-CH_2-$	gluconapoleiferin			●

Discussion

The quantitative inheritance of the content of sinigrin and progoitrin in Brussels sprouts was studied in terms of broad sense - and narrow sense heritability. These parameters indicate respectively the stability of quantitative traits in various environments and the influence of genotype on their expression. The broad sense heritability for progoitrin and sinigrin was found to be 0.77 and 0.79 respectively. Heaney & Fenwick (1980a) observed similarly high correlations between the content of the glucosinolates sinigrin and progoitrin of cultivars at 5 different locations. In our experiments much higher peak values were observed in the F1-hybrids for sinigrin (this paper: 3.2 g.kg^{-1}; Heaney & Fenwick: 1.6 g.kg^{-1}) and progoitrin (this paper: 3.5 g.kg^{-1}; Heaney & Fenwick: 1.0 g.kg^{-1}) compared to the results of Heaney & Fenwick (1980a). These differences might be explained by the population size of F1-hybrids used in the corresponding experiments. Based on probability a peak value for sinigrin and progoitrin is more likely to be found in a population of 150 F1-hybrids (our trials) than in a trial with 22 cultivars. The high broad sense heritability values indicate that the content of sinigrin and progoitrin of cultivars is relatively constant at

different production sites which greatly differ in soil type and other not specified environmental conditions.

For the production of sprouts with a predictably acceptable flavour, a constant low sinigrin and progoitrin content under various environmental conditions would be ideal. The relatively small environmental variance, between 21 and 35% of the phenotypic variance, is still substantial enough to conclude that cultivars with a rather high glucosinolate content can be unacceptable when cultivated in environments that promote glucosinolate accumulation. In this study, the cultivars were grown at different sites with different soil types and fertilized to guarantee optimal yield. In practice, the variation in soil type and fertilization regime between production sites is greater, resulting in considerable variation in the glucosinolate content of sprout F1-hybrids. Our previous results already indicated the necessity of breeding for cultivars with a low glucosinolate content which, despite the influence of environmental conditions, never accumulate a sum of sinigrin and progoitrin that exceeds the critical acceptance level of 2.2 g.kg^{-1} for consumers (Van Doorn et al., 1998a).

The biosynthesis routes leading to sinigrin and progoitrin predominate in Brussels sprout cultivars, as their glucosinolate content is mainly composed of these two glucosinolates (van Doorn et al., 1998c; Heaney & Fenwick, 1980a). The narrow sense heritability values for sinigrin and progoitrin are respectively 0.72 and 1.09. These high narrow sense heritability values indicate that, notwithstanding the large number of steps in the biosynthesis route, significant progress can still be made via selection for multigenic traits. The high values might indicate that only a few specific steps in the biosynthesis route are crucial for the content of sinigrin and progoitrin. The high narrow sense heritability for progoitrin of 1.09 (see Figure 3), and the relative high progoitrin content of the F1-hybrids at De Schermer (see Figure 2) suggests that progoitrin synthesis is dominant over sinigrin synthesis in specific situations.

One of the aims of our study was to develop Brussels sprout F1-hybrids with low contents of sinigrin and progoitrin. The results of this paper show a lack of correlation between the sinigrin and

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progoitrin content of Brussels sprout lines and F1-hybrids underlining the need to select for a low content of both glucosinolates. Sinigrin and progoitrin originate from L-homomethionine and L-dihomomethionine respectively (e.g. Bennett et al., 1996), glucosinolate precursors which differ in one extension step. The existence of three clusters of parental lines and F1-hybrids with respectively C_3, C_3+C_4 and $C_3+C_4+C_5$ aliphatic glucosinolates (see Table 2) suggests an important role for the enzymes which catalyze the side chain extension of the amino acid precursors of these aliphatic glucosinolates. These enzymes seem to distribute the extended methionine precursors for the biosynthesis of C_3, C_4 and C_5 aliphatic glucosinolates in Brussels sprouts. Based on the results of table 2, we postulate the existence of three elongation enzymes in Brussels sprouts for the conversion of methionine into respectively L-homomethionine, L-dihomomethionine (2-amino-6-thiocaproic acid) and L-trihomomethionine (2-methyl-7-thioheptanoic acid), the precursors of C_3, C_4 and C_5 aliphatic glucosinolates. Our hypothesis regarding the existence of multiple side chain extension enzymes in Brussels sprouts is supported by observations of other groups. In *Brassica napus* three elongation loci are also found which regulate the presence or absence of C_3, C_4 and C_5 glucosinolates (Magrath et al., 1994). In *Arabidopsis thaliana* two loci with an involvement in the side chain extension of methionine are observed, one regulating the conversion from C_3 to C_4 precursors (Mithen et al., 1995). The other is involved in the biosynthesis of C_6 to C_8 precursors (Haughn et al., 1991). Neither the side chain extension enzymes nor the genes that code for their expression have been characterized as yet. According to Glover et al. (1988), the first elongation step of methionine in *Brassica carinata* is catalyzed by the enzyme methionine-glyoxylate aminotransferase. In the various Brassicas many MGAT isozymes have been isolated which are, however, not yet characterized for substrate specificity (Chapple et al., 1990).

Many enzymes of the biosynthesis route of methionine derived glucosinolates have been characterized in recent years. The conversion of L-dihomomethionine (precursor of progoitrin) into its corresponding aldoxime is catalyzed by a flavin-containing

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monooxygenase (FMO) (Bennett et al., 1995 & 1996). The enzyme responsible for the conversion of L-homomethionine, the precursor of sinigrin into its corresponding aldoxime has not been identified and presumably is not a FMO. FMO enzymes are very substrate specific, as L-methionine derived amino acid dependent FMOs are not inhibited by L-TRP or L-Phe amino acid glucosinolate precursors (Bennett et al., 1995). The L-dihomomethionine dependent FMO, however, is competitively inhibited by L-trihomomethionine (Bennett et al., 1996). Magrath et al. (1993) have identified a locus in *Brassica napus* which regulates the production of C_3 glucosinolates including sinigrin. The enzyme(s) which catalyze the formation of thiohydroximates from aldoximes have not yet been isolated and characterized. The enzyme UDP-glucose:thiohydroxymate glucosyltransferase (Jain et al., 1989; Reed et al., 1993) catalyzes the attachment of the glucose moiety to the thiohydroximate group. This enzyme possesses a high specificity for the thiohydroximic functional group but little specificity for the associated side-chain groups (Reed et al., 1993). The introduction of the sulphate moiety to complete the glucosinolate is catalyzed by the enzyme 3'-PAPS-5'-phosphosulphate:desulphoglucosinolate sulphotransferase (Glendening & Poulton, 1988; Jain et al., 1989 & 1990). The biosynthesis route described yields glucosinolates with a variable chain length and a terminal thiomethyl-group. The side-chains of these glucosinolates can be further converted in various ways to give new glucosinolates with modified side-chains (Mithen et al., 1995; Parkin et al., 1994; Giamoustaris & Mithen, 1996). In Brussels sprouts side chains of the aliphatic glucosinolates are extensively modified as the sum of aliphatic glucosinolates is mainly comprised of sinigrin and progoitrin (van Doorn et al., 1998c).

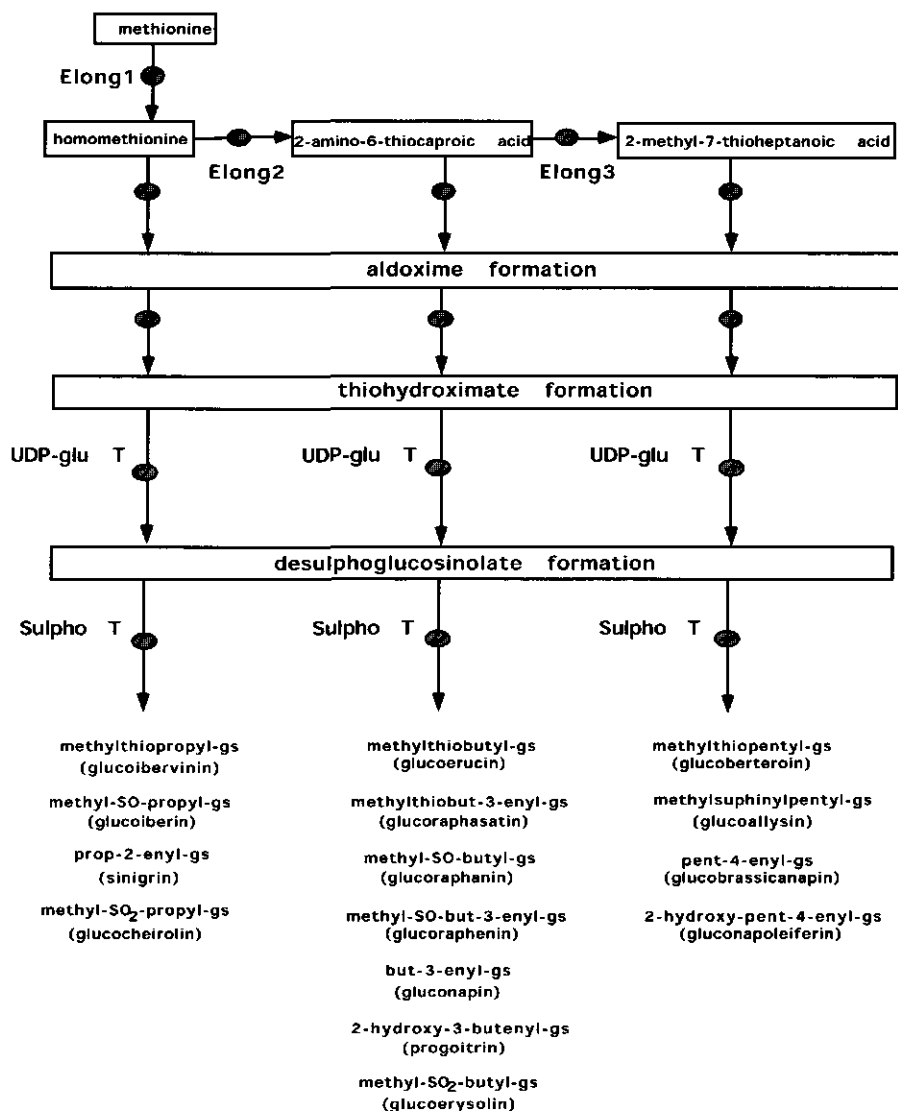*Figure 5.* Model for the biosynthesis of methionine derived aliphatic glucosinolates in Brassica vegetables. The trivial names of the glucosinolates are presented between brackets. Abbreviations: Elong = methionine elongation enzyme, UDP-glu T = UDP-glucose:thiohydroximate glucosyltransferase, Sulpho T = 3'PAPS-5'-phosphosulphate:desulphoglucosinolate sulphotransferase, gs = glucosinolate.

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Based on the information from this paper and the work of the groups cited a general biosynthesis route for methionine derived glucosinolates can be described. Figure 5 gives a schematic overview of the biosynthesis routes which lead to the methionine derived glucosinolates identified in Brassica crops. The glucosinolates with various types of side chains are presented at the bottom of Figure 5.

Selection for low contents of sinigrin and progoitrin was successfully applied in the breeding program. Reduction of the content of both glucosinolates to levels as low as 0.25 g.kg^{-1} is achievable in a couple of inbred generations. It is tempting to speculate that such a low content have a negative effect on the field performance of Brussels sprout cultivars. Many degradation products of aliphatic glucosinolates have anti-microbial activity towards non-host-specific pathogens (Drobnica et al., 1967; Weuffen et al., 1967a and 1967b). Glucosinolate-derived degradation products are lethal to nematodes (Lazzeri et al., 1993) and protect plants from slug damage (Glenn et al., 1990). Non-host specific insects (generalists) are repelled by glucosinolates (Erickson & Feeny, 1974) but host-specific insects (specialists), such as flea beetles and cabbage white butterflies, are attracted (Giamoustaris & Mithen, 1995). Degradation products of glucosinolates are reported to have antifungal activity towards *Peronospora parasitica* (Greenhalgh & Mitchell, 1976) *Leptosphearia maculans* (Mithen et al., 1986) and *Alternaria brassicae* (Doughty et al., 1991), all host specific fungi for Brassicas. A high glucosinolate content discourages grazing by pigeons (Giamoustaris & Mithen, 1995). A low sinigrin and progoitrin content had, however, no drawbacks on the field performance of "low" cultivars in terms of yield and standing ability.

The results of this paper show that breeding for a low sinigrin and progoitrin content becomes relatively simple once reliable methods are available to screen breeding programs on a large scale for such traits. The aim of the study to develop agronomically acceptable Brussels sprout cultivars with a low sinigrin and progoitrin content has been achieved. New F1-hybrids, such as Maximus and Helemus, which combine a good

flavour, optimal field performance and a high production of sprouts with a nice appearance prove that it is possible to breed for optimal product quality in Brussels sprouts using an integrated approach.

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Chapter 6
Soil sulfate, rainfall and root architecture influence the content of the glucosinolates sinigrin and progoitrin in Brussels sprouts

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Abstract

The sinigrin and progoitrin content of Brussels sprout cultivars is affected by the initial soil SO_4^{2-} concentration and cumulative rainfall. Based on differences in response to variation of these parameters individual cultivars can be grouped into three classes. Group 1 cultivars are unaffected by the initial soil SO_4^{2-} concentration and cumulative rainfall and maintain a constant glucosinolate content. The glucosinolate content of group 2 is positively and inversely correlated to respectively the soil SO_4^{2-} and rainfall; the content of group 3 is determined by an interaction between these environmental factors. The observed responses of the three groups of cultivars are determined by their root architecture. The sinigrin and progoitrin content in sprouts of most cultivars is related to the seedling cumulative lateral root length in laboratory germination tests. Group 1 cultivars have column-shaped root systems with regular lateral root formation along the tap root providing a constant SO_4^{2-} uptake. Group 2 cultivars have V-shaped root systems and predominantly take up SO_4^{2-} via superficial roots in the top soil layer. Group 3 cultivars have Y-shaped superficial lateral root formation and only take up SO_4^{2-} from the top soil layer. Our studies demonstrate that the accumulation of glucosinolates in Brussels sprouts is affected by root architecture parameters.

Introduction

Brussels sprouts is a nice example of a vegetable which is not appreciated equally by consumers. The flavor of Brussels sprouts is not acceptable to consumers when the sum of the glucosinolates sinigrin and progoitrin is higher than 2.2 g kg^{-1} fresh weight (van Doorn et al., 1998a). Sinigrin has a pronounced bitter taste in the native form, while progoitrin becomes bitter after enzymatic hydrolysis by the enzyme myrosinase (Griffiths and Fenwick, 1984; Fenwick et al., 1983). A new protocol for the preparation of Brussels sprout samples, specific ELISA assays for the glucosinolates sinigrin and progoitrin (van Doorn et al., 1998b) and an improved method for the determination of the glucosinolate content (van Doorn et al., 1998c) have opened up the opportunity to determine glucosinolates on a large scale. The methods have been applied to study the influence of phenotype and environment on the content of both glucosinolates in the Brussels sprouts breeding program.

In a previous study we have shown that the genotype plays a dominant role in the determination of the content of sinigrin and progoitrin in Brussels sprout cultivars (van Doorn et al., 1998d). In the same study, the broad sense heritability values for sinigrin and progoitrin indicated that the environment also has a significant influence. Environmental factors such as production site (Heaney and Fenwick, 1980; van Doorn et al., 1998d), soil composition and the amount and type of fertilizers (Heaney et al., 1983) clearly affect the glucosinolate content and composition of Brussels sprout cultivars. In general, Brassicas are vegetables with a relatively high sulfur requirement during their growth and development (Marschner, 1995). Sulfur is mainly taken up as SO_4^{2-} and then predominantly incorporated in sulfur-containing secondary metabolites such as glucosinolates and amino acids like S-methyl-cysteine sulfoxide (Schnug, 1993). In rapeseed, the seed and straw glucosinolate content increases at higher sulfur applications (Booth et al., 1991; Josefsson, 1971; Nuttall and Ukrainetz, 1991; Withers and O'Donnell, 1994). In crops such as white mustard, white cabbage and radish the production of glucosinolate-derived volatiles is directly related to the

concentration of SO_4^{2-} the plants were raised on (Freeman and Mossadeghi, 1972). These findings suggest that the observed variation in glucosinolate content of Brussels sprout F1-hybrids and their corresponding parental lines grown at different production sites is greatly influenced by the variation in SO_4^{2-} availability in the soil.

The concentration of SO_4^{2-} in soils is mainly determined by 4 different factors: -1- the application of SO_4^{2-} via various fertilizer formulations (Marschner, 1995), -2- the rate of SO_4^{2-} leaching by rain (Dijksterhuis and Oenema, 1990), -3- the rate of mineralization of SO_4^{2-} from organic matter in the soil (Eriksen et al., 1995) and -4- the rate of SO_4^{2-} formation from air-borne industrial SO_3. In general the SO_4^{2-} originating from fertilizers comprises the major part of the soil inorganic sulfur content. Therefore, excess uptake of SO_4^{2-} by Brussels sprout cultivars is expected to result in both a high total glucosinolate content and enhanced levels of specific glucosinolates. The SO_4^{2-} uptake by roots of plants is catalyzed by SO_4^{2-} uptake carriers (Lappartient and Touraine, 1996) whose activity is upregulated upon SO_4^{2-} starvation. Recently a proton/SO_4^{2-} cotransporter has been cloned and characterized in *Arabidopsis thaliana* (Takahashi et al., 1997).

The aim of this study is to determine the influence of the soil SO_4^{2-} concentration and cumulative rainfall on the content of sinigrin and progoitrin of a selection of commercial Brussels sprout cultivars at various production sites in consecutive years. The response of the sinigrin and progoitrin content of cultivars to variations in the former factors is studied as a function of root architecture and SO_4^{2-} uptake parameters. The definition of the key taste-affecting environmental factors and their control during the Brussels sprout production season are, along with the cultivar choice, the prerequisites for the production of sprouts with a reliable taste and glucosinolate content.

Materials and methods

Trials with Variable Soil Sulfate Concentration at Various Production Sites

The Brussels sprout cultivars Cyrus, Icarus, Kundry, Philemon, Adonis, Ajax and Lauris (all from Novartis Seeds BV) were grown in 1994 in the assessment trials for the evaluation of the field performance at the locations Kloosterburen (province Groningen), Enkhuizen, De Schermer and Hazerswoude in The Netherlands and at location Staden, Belgium, to study the impact of soil SO_4^{2-} concentration on their content of sinigrin and progoitrin. To validate the relationships between the soil SO_4^{2-} concentration observed at various locations and the sum of sinigrin and progoitrin of cultivars, as observed in 1994, the cultivars Adonis, Cyrus, Exodus, Genius, Helemus, Icarus, Louis, Maximus and Philemon (all from Novartis Seeds BV) were grown for the same purpose in Enkhuizen, two locations near Leens in the province Groningen (locations Groningen 1 and 2 in this paper), Hazerswoude and De Schermer in 1996. The cultivars were planted in the beginning of May and sprouts were harvested in the end of December. Samples were prepared and subsequently determined for the content of sinigrin and progoitrin according to the methods of Van Doorn et al. (1998b). Representative soil samples were collected from the 0 to 30 cm soil layer, the standard depth for fertilizer recommendations, at the various locations in the beginning of June for the determination of the SO_4^{2-} concentration at an early phase of crop development when the Brussels sprout plants had about 8 to 10 true leaves. Soil samples were extracted with water (soil:water = 1:2, v/v) and the filtrates obtained were subsequently determined for the concentration of SO_4^{2-} by the BLGG (Laboratory for Soil and Crop Research) in Naaldwijk (NL) and expressed as mM.

In both seasons, the trial fields were dressed with fertilizer in March (Ca/P/K/Mg/S) and April (N) according to the recommendations of the BLGG based on the analysis results of soil samples early in the season. Depending on the recommendations and the fertilizer composition, between 0 to 200 kg.ha^{-1} S was applied at the various fields. The variation in

soil type between the different locations, e.g. sand in Groningen and heavy clay in De Schermer, and the variable composition of the fertilizer dressings provided variation in soil SO_4^{2-} concentration.

Monitoring of the Soil Sulfate Concentration during a Production Season

The soil SO_4^{2-} concentration at location De Schermer was measured during a growing season to study its dynamics under the influence of rainfall and SO_4^{2-} uptake by a Brussels sprout crop. The experiment was also conducted to find the optimal sampling time of soil samples for the measurement of the soil SO_4^{2-} concentration with a predictive value for the sum of sinigrin and progoitrin of Brussels sprout cultivars. Representative soil samples from the 0 to 30 cm soil layer were collected in the trial field at weekly intervals and determined for the concentration of SO_4^{2-} according to the Spectroquant method (Merck, Germany).

Successive Trials with Recording of Cumulative Rainfall and Soil Sulfate Concentration at Location De Schermer

Between 1994 and 1997, nineteen Brussels sprouts cultivars, all from Novartis Seeds BV, were grown at location De Schermer as part of the regular breeding program with the aim of studying the influence of cumulative rainfall and soil SO_4^{2-} concentration on their sinigrin and progoitrin content. The growth period of the cultivars and the protocols for the collection of sprout samples, sample preparation, determination of sinigrin and progoitrin in sprout samples, the collection of soil samples and the determination of the soil SO_4^{2-} concentration were the same as for the trials in the former paragraphs. Each year, 932 kg ha^{-1} of Ca/P/K/Mg/S-based blended fertilizer was applied during the first or second week of March, followed by an application of 700 kg ha^{-1} N-based blended fertilizer at the end of April just before the crop was planted. According to the fertilizer specifications, a total of 54 kg S ha^{-1} was applied every season. Daily rainfall was recorded (in mm) before and during the production period by a local weather station every year.

Screening of Root Architecture Parameters of Seedlings of Brussels Sprouts Cultivars

Seeds from the Brussels sprout cultivars of the previous paragraph were disinfected by a ten min soak in 1% (v/v) sodium hypochlorite, extensively washed with sterilized demineralized water, and sown at a rate of twenty seeds per cultivar on plain 1% (w/v) water agar in 20*10*5 cm (length*width*height) white polycarbonate boxes with a transparent lid. The seeds were allowed to germinate in darkness for two days at 20 ºC, and subsequently exposed to conventional fluorescent light tubes (Philips TL33, 10.000 lux) in a 16-h light and 8-h darkness regime at 20 ºC. One week after sowing the root systems of ten uniform seedlings per cultivar were evaluated for the following root architecture traits: tap root length (mm), number of lateral roots (No.), cumulative length of lateral roots (cm), the shape of the root system (V-shape, Y-shape or column shape) and the distribution of lateral roots over the tap root. Root architecture traits are expressed as the mean of ten seedlings.

Sulfate Uptake Rate of Brussels Sprouts Cultivars in a Hydroponic System

The SO_4^{2-} uptake rate of Brussels sprouts cultivars was studied with seedlings of five weeks in age which were raised in a hydroponic system with a conventional nutrient solution and two levels of SO_4^{2-}. Seeds from Brussels sprouts cultivars were sown in duplicate on trays with rockwool plugs (Cultilene, Naaldwijk, The Netherlands, 240 plugs per tray) and covered with Vermiculite. The cultivar SG2106 was not included in the experiment due to the lack of seeds. The first tray, containing eighteen cultivars (twelve seeds per variety) was watered with a conventional nutrient solution containing 0.2 mM SO_4^{2-}, a concentration which should stimulate the expression of SO_4^{2-} uptake carriers in the root system (Lappartient and Touraine, 1996). The duplicate tray with the same cultivars was watered with the same nutrient solution containing 1.6 mM SO_4^{2-} which should suppress the expression of SO_4^{2-} uptake carriers. Apart from 0.2 or 1.6 mM SO_4^{2-}, the nutrient solution also comprised of 1 mM NH_4^+ , 8 mM K^+, 3.2 mM Ca^{2+}, 1.6 mM Mg^{2+}, 14.4 mM NO_3^-, 1 mM PO_4^{3-}, 15 μM Fe^{2+}, 7 μM Mn^{2+}, 20 μM B^+, 0.5 μM Cu^{2+} and 0.5

μM Mo^{2+} and adjusted at pH = 5.6. The seeds were allowed to germinate in darkness and 18 °C for three days and than exposed to conventional fluorescent light tubes (Philips TL33, 10.000 lux) in a 16-h light and 8-h darkness regime and the same temperature. After germination, the trays with seedlings were fed with the corresponding nutrient solutions until saturation every second day. The seedlings were grown for five weeks until the stage with four true leaves and subsequently used for the SO_4^{2-} uptake experiments.

The SO_4^{2-} uptake rate of seedlings raised on respectively 0.2 and 1.6 mM SO_4^{2-} was determined on the basic nutrient solution containing 3.2 mM SO_4^{2-} for a period of one day. Seedlings from the cultivars Kundry and Ariston were not included in the uptake experiments due to a poor germination. Five seedlings per cultivar per treatment were quickly rinsed in the nutrient solution with 3.2 mM SO_4^{2-} and put in plastic 50-mL jars containing twenty five mL of the same nutrient solution. The jars with seedlings were placed on a rotation mixer and gently mixed at a rate of 150 rpm. The SO_4^{2-} uptake of seedlings was monitored in 250 μL samples of nutrient solution which were collected from the jars at 30 min, 2 h 15 min , 4 h, 6 h, 9 h and 22 h 30 min after the start of the experiment. The experiment was then finished. The consumption of nutrient solution by the cultivars was recorded in order to calculate the SO_4^{2-} uptake rate. The seedling weight of five seedlings was measured to calculate the SO_4^{2-} uptake rate of cultivars per unit fresh weight. The seedling weight of five seedlings also was used to extrapolate the maximal SO_4^{2-} uptake rate of cultivars in the field at optimal SO_4^{2-} availability.

Statistics

The influence of cultivar, site and season on the content of sinigrin and progoitrin of cultivars was determined by an analysis of variance (factorial ANOVA). Significant differences between cultivars are expressed as least significant difference (LSD) values (at p = 0.05). The content of glucosinolates and the concentration of the soil SO_4^{2-} have been determined in duplicate. Only duplicate readings differing 0.020 absorbance unit or less were used for calculations. These criteria for duplicate readings result in small and constant standard deviations for

measurements and are not presented in the tables of this manuscript.

The influence of rainfall and soil SO_4^{2-} concentration on the content of the glucosinolates sinigrin and progoitrin of Brussels sprouts cultivars was evaluated by comparison of annual differences in the sum of both glucosinolates of cultivars (Δsum between years), cumulative rainfall (Δmm rain between years) and soil SO_4^{2-} concentration (ΔSO_4^{2-} between years). Rainfall, soil SO_4^{2-} concentration and the content of sinigrin and progoitrin were measured from 1994 up to 1997 and consequently annual differences for these factors could be calculated between, respectively, 1994-1995, 1994-1996, 1994-1997, 1995-1996, 1995-1997 and 1996-1997. The parameters are expressed on a continuous scale and can adopt negative or positive values depending on the data pair compared. We have chosen for such a design with derived data for an optimal extraction of information from 4 years of experiments. Thus, six in stead of 4 data points per attribute are available for the (groups) of cultivars to study putative mutual relationships. On the one hand two out of six degrees of freedom are used for the calculation of annual differences and lost for regression analysis, on the other hand the extended data set is giving a more consistent view with respect to the mutual relationships between the attributes than a data set with 4 points. Mutual relationships between the former parameters were calculated with (multiple) regression analysis and significance levels are indicated where appropriate.

Assumptions used for the Calculation of the Sulfur or Sulfate Content in Plants and Soil

1: Dry and fresh weight production of Brussels sprouts

The dry weight of Brussels sprout plants is on average 15% (w/w) of the fresh weight (Souci, Fachman and Kraut, 1989). In practice 33,000 plants are grown per hectare which produce on average a total fresh weight of 74.3 ton (sprout buttons: 0.75 kg/plant; plant without buttons: 1.5 kg/plant) including 25 ton sprouts.

2: S-content of glucosinolates

The average M_r of sinigrin and progoitrin is, after Carlson et al. (1987), 457. The glucosinolates sinigrin and progoitrin contain 2

atoms S per molecule (M_r per S = 32), which is 14% (w/w) S. A glucosinolate content of 100 mg 100 g^{-1} in Brussels sprout cultivars corresponds with 10.4 kg S ha^{-1} according to the assumptions of the previous paragraph.

Results and discussion

The Influence of Growth Site on the Glucosinolate Content of Cultivars

According to the broad sense heritability values we observed previously (Van Doorn et al., 1998d), the variation in the content of sinigrin and progoitrin of Brussels sprout cultivars is determined for 30 to 40% by environmental factors. In order to identify these environmental glucosinolate content-determining factors seven Brussels sprout cultivars were grown in 1994 at five different locations in The Netherlands and Belgium. In table I the content of sinigrin, progoitrin and the sum of both glucosinolates of the cultivars at the different locations are presented. The cultivars were selected to represent the full range of glucosinolate contents and compositions normally found. The cultivars of table I had different contents of sinigrin and progoitrin regardless of the production location (factorial ANOVA, p<0.01). On average Lauris had the highest sum of sinigrin and progoitrin over the locations, whereas Icarus had the lowest. The average content of sinigrin and progoitrin of all cultivars was significantly different between locations suggesting that the production site had an effect on the content of glucosinolates (factorial ANOVA, p<0.01). The highest average sum of sinigrin and progoitrin was at Enkhuizen and the lowest at Hazerswoude. The observed differences in the content of sinigrin and progoitrin of cultivars between locations are expected to be determined by site-specific differences in environmental conditions. The average sinigrin content of the cultivars at the different locations was correlated with the soil SO_4^{2-} concentration (0 to 30 cm depth), as determined in mid June at an early stage of crop development (r=0.91, p=0.10, n=4).

Table I. The content of sinigrin, progoitrin and the sum of both glucosinolates in Brussels sprouts cultivars at various locations in 1994. Abbreviations: sin = sinigrin, prog = progoitrin, sum = progoitrin + sinigrin, all in mg/100g; mean locations = the mean content of sinigrin, progoitrin and sum of sinigrin and progoitrin of all cultivars over the locations, nd = not determined nm = not measured at this location. At the bottom of the table mM SO_4^{2-} denotes the soil SO_4^{2-} concentration in the 0 to 30 cm soil layer at the start of the season.

cultivar	Groningen			Enkhuizen			De Schermer			Hazerswoude			Staden			mean locations		
	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum
Cyrus	91	25	116	117	32	149	94	28	122	87	23	110	102	29	131	98	27	125
Icarus	86	31	117	90	28	118	73	20	93	69	18	87	59	18	77	75	23	98
Kundry	55	71	126	65	39	104	44	54	98	33	42	75	nm	nm	nm	43	58	101
Philemon	74	79	153	72	97	169	64	74	138	53	66	119	57	57	114	64	75	139
Adonis	65	55	120	87	75	162	36	38	74	30	31	61	40	36	76	52	47	99
Ajax	144	35	179	144	57	201	106	37	143	107	18	125	119	26	145	124	35	159
Lauris	86	95	181	99	127	226	80	95	175	nm	nm	nm	78	78	156	86	99	185
mean	86	53	139	92	69	161	71	49	120	63	33	96	76	41	117			
mM SO_4^{2-}		nd			0.7			0.4			0.3			0.2				

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The observed correlation was pulled to a high extent by particular cultivars. The sum of sinigrin and progoitrin of the cultivars Icarus, Philemon and Lauris was highly correlated with the soil SO_4^{2-} concentration (Fig. 1A). Similar findings have been observed for single cultivars from other crucifers (Freeman and Mossadeghi, 1972; Marschner, 1995). For the other cultivars of table I lower, even non-significant, correlations were observed suggesting that the glucosinolate content of cultivars not always was determined by the variation in soil SO_4^{2-} concentration.

In 1996, nine sprout cultivars were grown at five locations in The Netherlands to confirm whether the observed relationship between the soil SO_4^{2-} concentration at an early stage of plant development and the sum of sinigrin and progoitrin at the point of harvest, as observed in 1994, was consistent in time. The average content of sinigrin, progoitrin and the sum of sinigrin and progoitrin of cultivars was significantly different between locations (ANOVA, $p < 0.01$) (Table II). Many cultivars in table II, such as Maximus and Helemus, were specifically bred for a low sum of sinigrin and progoitrin, regardless of the environment they have to grow in. As a consequence, a rather low correlation was observed between the average sum of sinigrin and progoitrin of the nine tested cultivars and the SO_4^{2-} concentration in the soil. The sum of both glucosinolates was, however, for Louis, Icarus and Philemon, again highly correlated with the soil SO_4^{2-} concentration. In figure 1B, typical relationships between the soil SO_4^{2-} concentration at different production sites and the sum of sinigrin and progoitrin are presented for these cultivars. The equations in figures 1A and B, which describe the relationships between the soil SO_4^{2-} concentration and the sum of sinigrin and progoitrin of the cultivars Philemon and Icarus, are different in 1994 and 1996. The marked difference in the soil SO_4^{2-} range in 1994 and 1996 likely is the reason why the former cultivars have different kinetics of glucosinolate accumulation over the years. The variable nature of the equation for specific cultivars indicates that additional, presumably environmental, factors such as the dynamics of the soil SO_4^{2-} play a role in the uptake and utilization of SO_4^{2-} by Brussels sprouts plants for the biosynthesis of these glucosinolates.

Dynamics of Soil Sulfate Content during the Growth Period

In figures 1A and B the sum of sinigrin and progoitrin of one group of cultivars is correlated with the soil SO_4^{2-} concentration at the start of the production season. Other cultivars, however, maintain a rather constant sum of sinigrin and progoitrin under the same conditions (see tables I and II). These results indicate that the sinigrin and progoitrin content of some cultivars is more sensitive for environmental fluctuations than that of others. The soil SO_4^{2-} concentration is positively affected by the mineralization rate of SO_4^{2-} from organic matter, the rate of SO_4^{2-} formation from industrial SO_3 and the application of SO_4^{2-} in the form of fertilizer blends (Marschner, 1995). Excessive rain stimulates SO_4^{2-} leaching (Dijksterhuis and Oenema, 1990), and is probably the main reason for the loss of SO_4^{2-} during the cultivation period.

The soil SO_4^{2-} concentration and relevant factors which might affect this parameter were evaluated during one Brussels sprouts production season. The soil S content (= soil SO_4^{2-} concentration * 3.10^6 * M_r of sulfur in kg ha^{-1}) in the 0 to 30 cm layer in relation to rainfall at weekly intervals is presented in figure 2. The soil S content started at 70 kg ha^{-1} in week 23, just after planting, and quickly went down to almost 0 kg ha^{-1} in week 31. Available SO_4^{2-} in the soil is apparently absorbed by the plants during early crop development or transported to the sub soil layer by rain. In week 32, 35, 41 and 43 four peaks in soil S content between 8 and 31 kg S ha^{-1} and a joint sum of almost 100 kg ha^{-1} were observed. Except for peak 1, which is coming up at the end of a rainy period, these peaks coincided with periods of rainfall suggesting that rain was involved in the liberation of SO_4^{2-} from SO_4^{2-} containing sources.

The amount of 100 kg S ha^{-1} liberated during the leaf abscission period is 30 kg S ha^{-1} higher than the initial 70 kg S ha^{-1} found in the beginning of June. S can also originate from S-deposition from rain, S-mineralization from soil organic matter during the

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Table II. The content of sinigrin, progoitrin and the sum of both glucosinolates in Brussels sprouts cultivars at various locations in 1996. Abbreviations: sin = sinigrin, prog = progoitrin, sum = progoitrin + sinigrin, all in mg/100g; mean locations = the mean content of sinigrin, progoitrin and sum of sinigrin and progoitrin of all cultivars over the locations, nm = not measured at this location. At the bottom of the table mM SO_4^{2-} denotes the soil SO_4^{2-} concentration in the 0 to 30 cm soil layer at the start of the season.

| cultivar	Enkhuizen			Groningen 1			Groningen 2			Hazerswoude			De Schermer			mean locations		
	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum
Adonis	57	63	120	81	80	161	56	64	120	78	71	149	57	69	126	66	69	135
Cyrus	91	26	117	112	45	157	126	39	165	99	29	128	81	40	121	102	36	138
Exodus	61	25	86	108	35	143	111	34	145	39	13	52	42	21	63	72	26	98
Genius	69	29	98	110	79	189	105	38	143	51	15	66	67	40	107	80	41	121
Helemus	42	23	65	64	45	109	90	39	129	35	20	55	33	28	61	53	31	84
Icarus	103	23	126	87	44	131	96	32	128	56	17	73	90	29	119	86	29	115
Louis	67	39	106	100	41	141	87	39	126	35	28	63	54	33	87	69	36	105
Maximus	42	29	61	68	44	112	60	30	90	41	45	86	37	18	55	50	31	81
Philemon	nm	nm	nm	90	128	218	nm	nm	nm	72	96	168	78	107	185	80	110	190
mean	67	30	97	91	60	151	92	39	131	56	37	93	60	43	103			
mM SO_4^{2-}	1.4			1.8			1.2			0.5			1.0					

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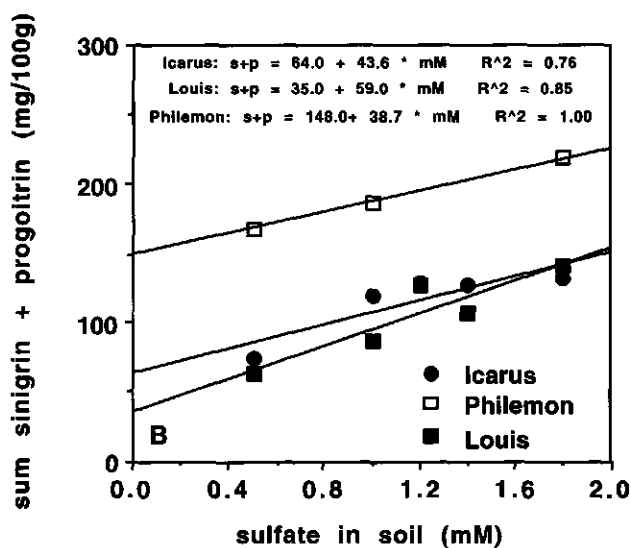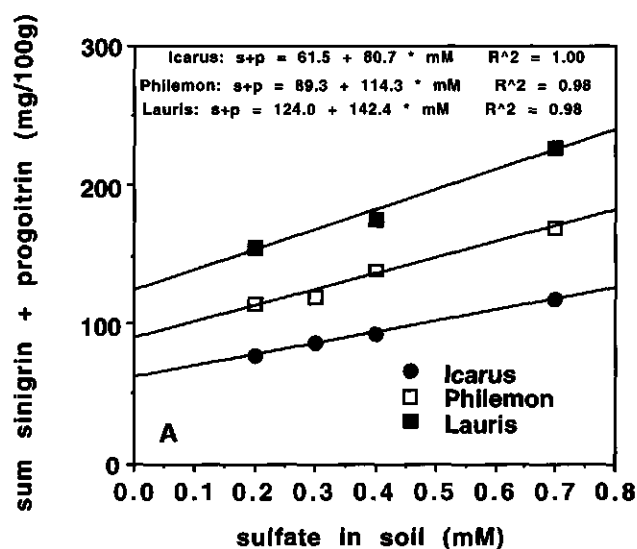
Figure 1. Relationships between the soil SO_4^{2-} concentration (in mM) at the start of the season and the sum of sinigrin and progoitrin (in mg/100g) of 3 different Brussels sprout cultivars at different production locations in 1994 (upper graph A) and 1996 (lower graph B). Abbreviations: s+p = sum of sinigrin and progoitrin, mM = millimolar.

growth period and evaporation-driven mass flow of SO_4^{2-} from sub soil layers (Marschner, 1995). S-mineralization from organic matter in the soil is reported to vary between 20.0 (Eriksen et al., 1995) and 29 kg S ha^{-1} year^{-1} (Goh and Nguyen, 1997) in the 0 to 30 cm soil layer and mainly takes place at higher temperatures in the summer (MacDonald et al., 1995). In the USA, S-deposition originating from rain varied between 1 and 9 kg S ha^{-1} year^{-1} in 1995 (http://nadp.nrel.colostate.edu/, 1995).

The peaks in soil SO_4^{2-} content were observed in the leaf abscission period, which is determined by plant age. Brussels sprout plants drop about 80% of their leaves prior to sprout production. The timing of leaf abscission and sprout production, for e.g. the cultivar Maximus, in relation to the soil SO_4^{2-} content is also presented in figure 2. The observed soil SO_4^{2-} peaks in the leaf abscission period seem to originate from organic matter derived SO_4^{2-} from dropped leaves which is washed out by rain. Brussels sprouts plants have two peaks of SO_4^{2-} uptake during the season. The first during an early phase of crop development, the second during the sprout production period. Liberated SO_4^{2-} from dropped leaves is for a significant part used during the latter period, either for biosynthesis of glucosinolates or for the synthesis of protein. Figure 2 demonstrates that the initial soil SO_4^{2-} concentration shortly after planting and rainfall are important factors for the availability of SO_4^{2-} during the growth period of Brussels sprout plants.

Influence of the Soil Sulfate Concentration and Cumulative Rainfall on the Sum of Sinigrin and Progoitrin of Cultivars

The impact of the soil SO_4^{2-} concentration and cumulative rainfall during the sprout production period on the sinigrin and progoitrin content of cultivars was studied in four consecutive years with nineteen cultivars at location De Schermer. In table III (left columns) the content of sinigrin, progoitrin and the sum of both glucosinolates for the cultivars through the years is presented. At the bottom of the table the soil SO_4^{2-} concentration and the cumulative rainfall are presented for the consecutive years.
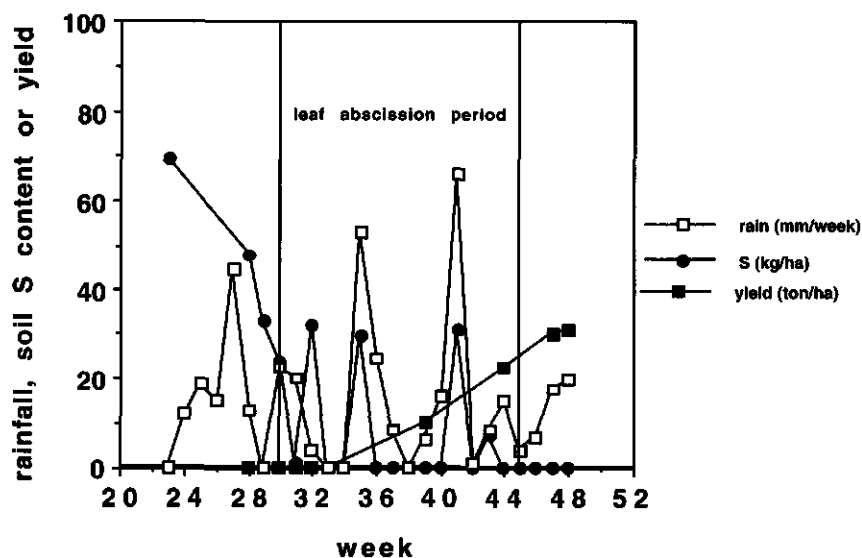
Figure 2. Relationships between the soil S content (in kg S/ha in the 0 to 30 cm soil layer), rainfall (in mm/week), and the cumulative sprout production of the cv Maximus (in ton/ha) in the season of 1997. The soil S content and rainfall were determined at the point of planting and at weekly intervals from week 28 onwards after removal of the crop protection foil.

The cultivars had significantly different contents of sinigrin, progoitrin and sum of both glucosinolates over the years (factorial ANOVA: sinigrin, $p = 0.0001$, LSD = 31; progoitrin, $p = 0.0001$, LSD = 21; sum, $p = 0.0001$, LSD = 48). The content of sinigrin and progoitrin and the sum of both glucosinolates of the cultivars was significantly different between the years (multiple factor ANOVA, $p<0.01$ for all glucosinolates). The sinigrin content of the cultivars is significantly mutually correlated between the years 95-96, 96-97 and 95-97 (r^2-values between 0.59 and 0.62), except for the combinations between the sinigrin values of the year 1994 and other years (r^2-values between 0.23 and 0.41). The excessive rainfall and the low soil SO_4^{2-} concentration in 1994 might be the reason for the absence of significant correlations. The observed correlation coefficient values agree well with our previous results (Van Doorn et al., 1998d).

Table III. The content of sinigrin, progoitrin, the sum of both glucosinolates and year to year differences in the sum of sinigrin and progoitrin, all in mg/100g, in Brussels sprouts cultivars at location De Schermer between 1994 and 1997. At the bottom of the table environmental glucosinolate content affecting factors and year to year differences in these factors are presented: mm rain = cumulative rainfall during the production period in mm, mM SO_4^{2-} = SO_4^{2-} concentration in the 0 to 30 cm soil layer in mM at the start of the season, Δmm rain = difference in cumulative rainfall between years in mm, ΔmM SO_4^{2-} = difference in soil SO_4^{2-} concentration between years in mM. Abbreviations: sin = sinigrin, prog = progoitrin, sum = sinigrin and progoitrin, all in mg/100g, mean sum = the mean sinigrin + progoitrin content of cultivars between 1994 and 1997, Δsum between years = difference in sum of sinigrin and progoitrin between years.

cultivar	1994			1995			1996			1997			mean sum	Δ sum between years					
	sin	prog	sum	sin	prog	sum	sin	prog	sum	sin	prog	sum	all years	94-95	94-96	94-97	95-96	95-97	96-97
Ilias	77	49	126	165	110	275	78	59	137	86	54	140	170	-149	-11	-14	138	135	-3
Maximus	29	12	41	41	23	64	52	29	81	46	37	83	67	-23	-40	-42	-17	-19	-2
Cyrus	85	19	104	164	44	208	124	41	165	157	52	209	172	-104	-61	-105	43	-1	-44
Kundry	40	57	97	44	58	102	47	71	128	54	71	125	113	-5	-31	-28	-26	-23	3
Philemon	50	68	116	86	89	175	77	101	178	80	103	183	163	-59	-62	-67	-3	-8	-5
Helemus	38	21	59	53	44	97	38	27	65	57	54	111	83	-38	-6	-52	32	-14	-46
Angus	65	23	88	81	41	122	86	47	133	70	47	117	115	-34	-45	-29	-11	5	16
Louis	50	26	76	69	45	114	82	33	115	104	60	164	117	-38	-39	-88	-1	-50	-49
SG 2116	53	51	104	39	81	120	55	79	134	48	80	128	122	-16	-30	-24	-14	-8	6
SG 2106	78	20	98	144	33	177	139	37	176	155	32	187	160	-79	-78	-89	1	-10	-11
Lauris	72	75	147	92	103	195	86	105	191	117	132	249	196	-48	-44	-102	4	-54	-58
Origus	87	40	127	132	44	176	98	41	139	103	25	128	142	-49	-12	-1	37	48	11
Ajax	131	21	152	136	46	182	115	27	142	104	33	137	153	-30	10	15	40	45	5
Exodus	59	31	90	79	19	98	57	18	75	60	25	85	87	-8	15	5	23	13	-10
Genius	34	28	62	81	22	103	96	42	138	79	27	106	102	-41	-76	-44	-35	-3	32
Adonis	55	47	102	82	67	149	77	61	138	38	34	72	115	-47	-36	30	11	77	66
Stephen	110	102	212	114	72	186	87	66	153	106	76	185	184	26	59	27	33	1	-32
Ariston	38	41	79	68	39	107	34	44	78	61	54	116	95	-28	1	-37	29	-9	-38
Tavernos	35	36	71	77	51	128	41	52	93	95	90	185	119	-57	-22	-114	35	-57	-92
mean year	63	40	103	93	53	146	77	52	129	87	56	143		-44	-27	-40	17	4	-13
mm rain	717			424			549			528									
mM SO_4^{2-}	0.4			1.4			1.0			0.8									
Δmm rain														293	168	189	-125	-104	21
ΔmM SO_4^{2-}														-1.0	-0.6	-0.4	0.4	0.6	0.2

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Comparable correlation coefficient values were observed for progoitrin between the years (data not shown). If cumulative rainfall and soil SO_4^{2-} concentration influence the sum of sinigrin and progoitrin of cultivars, then variations in these factors over the years should be mutually correlated. In the right columns of table III, differences in the sum of both glucosinolates of cultivars (Δsum between years), cumulative rainfall (Δmm rain between years) and soil SO_4^{2-} concentration (ΔSO_4^{2-} between years) are presented for all possible six year to year combinations. The factors are expressed on a continuous scale and can adopt negative or positive values depending on the data pair compared. In figure 3 the relationships between the annual differences in the average sum of glucosinolates of cultivars (horizontal axis), cumulative rainfall (left vertical axis) and soil SO_4^{2-} concentration (right vertical axis) are shown. From figure 3 it is clear that a low cumulative rainfall (negative Δrain values) and a high soil SO_4^{2-} concentration (positive ΔSO_4^{2-} values) stimulate a high average sum of sinigrin and progoitrin (positive Δsum values) in cultivars. The soil SO_4^{2-} concentration at the start of the cultivation period, which is mainly determined by the amount of fertilizer applied, and cumulative rainfall during the growing season obviously are independent factors. The role rain plays in the lowering of the glucosinolate content of cultivars by stimulation of SO_4^{2-} displacement to the sub soil layers seem to be in contrast to its capacity to mobilize SO_4^{2-} from abscised leaves after the S-mineralization process (cf. figure 2). Rainfall primarily seems to determine the balance between SO_4^{2-} uptake by the crop and leaching of SO_4^{2-} to the sub soil layers during early crop development. Rainfall is in the second place also the driving force for SO_4^{2-} release from abscised leaves. The root systems of the various Brussels sprout cultivars apparently have a relative low SO_4^{2-} uptake capacity allowing the mass flow of non-absorbed SO_4^{2-} to sub soil layers during rainfall. The soil SO_4^{2-} concentration in June (week 23, figure 2) is positively correlated to the average sum of sinigrin and progoitrin of cultivars, demonstrating that SO_4^{2-} availability is a key condition for the biosynthesis of glucosinolates. The parameters on the vertical axes are co-incidentally inversely related as almost all

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the rain fell after the determination of the soil SO_4^{2-} concentration in mid June. In practice SO_4^{2-}-containing fertilizer is applied at the beginning of March while the cultivars are planted between the middle and the end of May.

The soil SO_4^{2-} concentration in the 0 to 30 cm layer in mid June is inversely related to the cumulative rainfall between the point of fertilizer application in March and soil analysis (SO_4^{2-} June = 2.0 - 0.14 * rainfall, r^2 = 0.73, p<0.10, rainfall data not shown). These results indicate a high rate of SO_4^{2-} flow to the sub soil layers between the application of fertilizer and the point of planting.
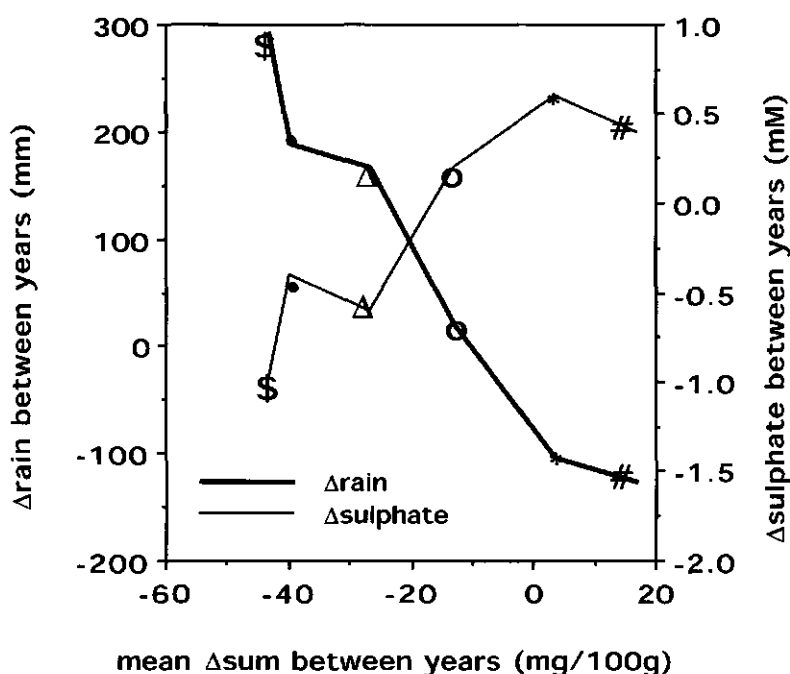

Figure 3. The relationships between the "Δsum of sinigrin and progoitrin between years" (in mg/100g, horizontal axis), "Δrain between years" (in mm, left vertical axis) and "Δ SO_4^{2-} between years" (in mM, right vertical axis). The data points of the year to year combinations for Δ rain and Δ SO_4^{2-} refer to: $ = 94-95, • = 94-97, Δ = 94-96, o = 96-97, * = 95-97 and # = 95-96.

Individual Responses of Cultivars to Variation in Soil Sulfate Concentration and Rainfall

In figure 3 the average response of the sum of progoitrin and sinigrin of the cultivars of table III to variations in soil SO_4^{2-} concentration and cumulative rainfall in consecutive years is presented. The individual responsiveness of cultivars to variations in these factors through the years is shown in table IV. The responsiveness of the sum of progoitrin and sinigrin of individual cultivars to variations in soil SO_4^{2-} concentration and cumulative rainfall was quantified with (multiple) regression analysis. Significant relationships are presented as the corresponding square (multiple) correlation coefficients. The decrease and increase in the sum of sinigrin and progoitrin of cultivars respectively per 100 mm rain and 1 mM SO_4^{2-}, as calculated with the equations of significant relationships, are indicative of the impact of soil SO_4^{2-} concentration and rainfall on the glucosinolate content of cultivars.

In table IV, three groups of cultivars are categorized, based on these individual relationships between the former factors. Group 1 comprises cultivars with a low to high sum of progoitrin and sinigrin, whose sum is not affected by variations in the soil SO_4^{2-} concentration and cumulative rainfall in consecutive years.

Group 2 comprises cultivars whose sum of sinigrin and progoitrin is responsive to variations in the soil SO_4^{2-} concentration and cumulative rainfall. Group 3 comprises cultivars whose sum of sinigrin and progoitrin is determined by an interaction between the soil SO_4^{2-} concentration and cumulative rainfall. The response of the sum of sinigrin and progoitrin of the cultivars of the 3 groups to variations in soil SO_4^{2-} concentration and cumulative rainfall is presented in figure 4. The cultivars Maximus and Kundry from group 1 have a constant low sum of progoitrin and sinigrin, independent of the soil SO_4^{2-} concentration and cumulative rainfall, and are ideal for the cultivation of sprouts with a good flavor (Van Doorn et al., 1998a). A cultivar such as Stephen is, on the same criteria, not suitable for this purpose.

The accumulation of sinigrin and progoitrin in cultivars can be viewed as possessing 3 rate limiting steps: -1- the uptake of

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Table IV: The responsiveness of the sum of sinigrin and progoitrin of Brussels sprouts cultivars to variations in soil SO_4^{2-} concentration at the start of the season and cumulative rainfall.

group	cultivar	sum prog+sin (mg/100g)	mr²	Δsum-Δrain			Δsum-ΔSO_4^{2-}		
				r²	sign.	Δsum rain mg/100 mm	r²	sign.	Δsum SO_4^{2-} mg/1 mM
group 1	Maximus	67	nr	0.28	ns	nr	0.30	ns	nr
	Exodus	87	nr	0.30	ns	nr	0.17	ns	nr
	Genius	102	nr	0.24	ns	nr	0.38	ns	nr
	Kundry	113	nr	0.02	ns	nr	0.00	ns	nr
	SG 2116	122	nr	0.23	ns	nr	0.34	ns	nr
	Stephen	184	nr	0.13	ns	nr	0.26	ns	nr
	mean	113	nr	0.20	nr	nr	0.24	nr	nr
group 2	Angus	115	nr	0.52	p<0.10	10	0.68	p<0.05	31
	Origus	142	nr	0.91	p<0.05	20	0.94	p<0.05	54
	SG 2106	160	nr	0.86	p<0.05	23	0.82	p<0.05	60
	Ajax	153	nr	0.77	p<0.05	14	0.74	p<0.05	37
	Philemon	163	nr	0.82	p<0.05	17	0.80	p<0.05	45
	Ilias	170	nr	0.89	p<0.05	60	0.83	p<0.05	157
	mean	150	nr	0.80	nr	24	0.80	nr	64
group 3	Helemus	83	0.97	0.96	p<0.01	71	0.96	p<0.01	166
	Ariston	95	0.94	0.94	p<0.01	59	0.92	p<0.01	140
	Adonis	115	1.00	0.98	p<0.01	72	1.00	p<0.01	257
	Louis	117	0.87	0.90	p<0.05	63	0.82	p<0.05	157
	Tavernos	119	1.00	1.00	p<0.01	130	1.00	p<0.01	325
	Cyrus	172	0.99	0.98	p<0.01	80	0.98	p<0.01	131
	Lauris	196	0.91	0.90	p<0.05	77	0.88	p<0.05	187
	mean	128	0.95	0.95	nr	79	0.93	nr	195

SO_4^{2-} by the root system (Marschner, 1995), -2- the incorporation of SO_4^{2-} in methionine , and -3- the biosynthesis of progoitrin and sinigrin from methionine (van Doorn et al., 1998d). The cultivars Maximus and Kundry of group 1, with a constant, low sum of sinigrin and progoitrin, either have a low SO_4^{2-} uptake by the roots or a low biosynthesis rate of glucosinolates from methionine. The lack of response of "low" cultivars to a more than threefold increase in soil SO_4^{2-} suggests that the SO_4^{2-} uptake in the roots is the limiting factor for the biosynthesis of both glucosinolates. The cultivar Stephen apparently has a high SO_4^{2-} uptake and a high biosynthesis rate as its sum of sinigrin and progoitrin is, regardless the environmental conditions, constantly high. The high SO_4^{2-} uptake by Stephen under any conditions suggests that the cultivar has a well-developed and deeply penetrating root system in the top and sub soil layers and/or a very efficient SO_4^{2-} uptake system. The sum of progoitrin and sinigrin of cultivars from group 2 responds to variations in the soil SO_4^{2-} concentration and cumulative rainfall. Rainfall and soil SO_4^{2-} concentration have opposite effects on the sum of sinigrin and progoitrin of the cultivars. Both environmental factors independently affect the sum of sinigrin and progoitrin of cultivars.
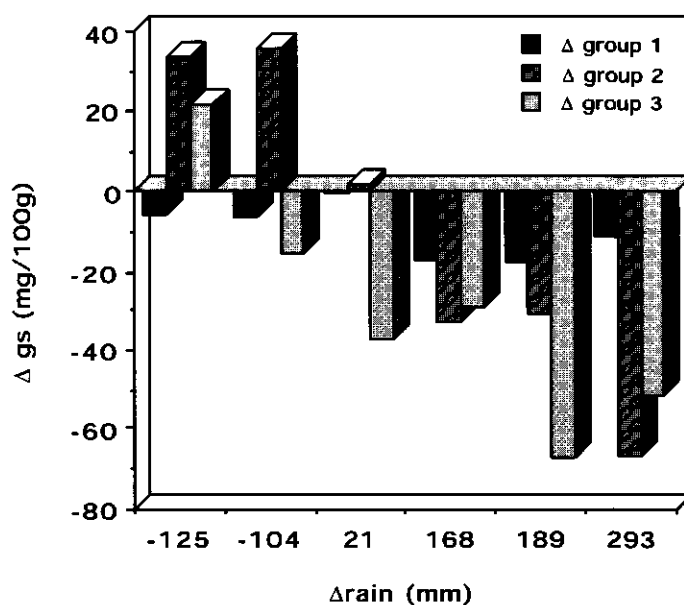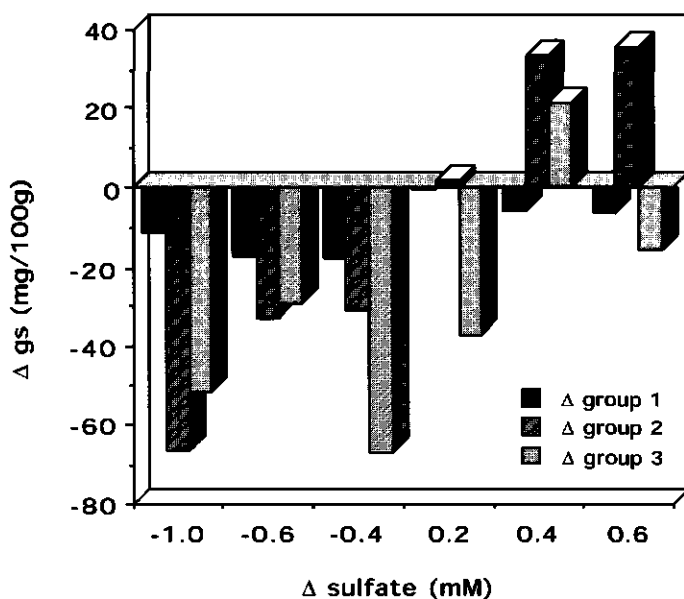
Figure 4. The response of the sum of sinigrin and progroitrin of Brussels sprout cultivars to year to year variations in the soil SO_4^{2-} and cumulative rainfall (see legend on next page).

Their influence on the sum of sinigrin and progoitrin can be calculated with simple linear regression. The cultivars have on average a high sum of progoitrin and sinigrin which can, however, be decreased to acceptable levels for consumers by reduced application of SO_4^{2-}-containing fertilizer and the selection of production sites with a low soil SO_4^{2-} concentration prior to planting of the crop.

A decrease of between 31 mg 100 g^{-1} (Angus) and 157 mg 100 g^{-1} (Ilias) per mM decrease in soil SO_4^{2-} concentration can be calculated for the cultivars of group 2, using the linear relationships (equations not shown) between the "Δsum of progoitrin and sinigrin" and "Δsoil SO_4^{2-} concentration" (see table IV). These values correspond to 31% and 92% of the average sum of progoitrin and sinigrin of respectively Angus and Ilias over the locations. The sum of sinigrin and progoitrin of the group 2 cultivars will be further reduced by excessive rainfall due to rain stimulated leaching.

The underlying mechanism of this reduction in glucosinolates is presumably the leaching or redistribution of SO_4^{2-} to lower soil layers beyond the reach of the root system of these cultivars. Reduction of the soil SO_4^{2-} concentration is the most effective strategy to decrease the glucosinolate content of the cultivars of group 2 (average decrease of 64 mg mM^{-1} SO_4^{2-}).

Legend to figure 4. The response of the sum of sinigrin and progroitrin (Δgs in mg/100g) of Brussels sprout cultivars from groups 1, 2 and 3 to year to year variations in the soil SO_4^{2-} concentration (Δsulphate in mM, upper panel) and cumulative rainfall (Δrain in mm, lower panel). The average response of the groups of cultivars to variation in soil sulphate and rainfall is calculated with linear regression analysis and the "Δsum between years", "Δmm rain" and "Δ mM SO$_4^{2-}$" data from table III. The bars which depict the relationships between the independent factors for groups 1 and 2 are the result of simple regression plots, those of group 3 the result of partial regression analysis. The bar values in the graphs, which present the partial regression relationships between the factors for group 3, represent the contribution of "Δmm rain" (lower graph) or "Δ mM SO$_4^{2-}$" (upper graph) to the "Δsum between years" after correction for the contribution of the other factor involved in the multiple regression calculations.

The sum of sinigrin and progoitrin of the cultivars of group 3 responds to variations in the soil SO_4^{2-} concentration and cumulative rainfall. Rainfall and the soil SO_4^{2-}, in interaction, affect the sum of sinigrin and progoitrin of group 3 cultivars. The influence of rainfall and soil SO_4^{2-} on the sum of sinigrin and progoitrin has to be calculated with multiple regression analysis. Moreover, the influence of soil SO_4^{2-} concentration and cumulative rainfall on the content of glucosinolates of cultivars is much more pronounced compared to the cultivars of group 2.

The sum of sinigrin and progoitrin of the cultivars of group 3 is on average threefold more sensitive to variations in rainfall and the soil SO_4^{2-} concentration than those of group 2 (rain: 24 and 79 mg 100 g^{-1} per 100 mm rain for respectively group 2 and 3; SO_4^{2-} : 64 and 195 mg 100 g^{-1} per mM SO_4^{2-} for respectively group 2 and 3). The observed decrease in the sum of sinigrin and progoitrin of the cultivars under the influence of rainfall and a low soil SO_4^{2-} concentration suggests that their root systems have a superficial and lesser reach, and a higher SO_4^{2-} uptake per unit root volume, than the cultivars of group 2. Rainfall is an unpredictable and therefore an unreliable tool for the reduction of the glucosinolate content of cultivars. The glucosinolate content of the cultivars of group 3 can also be effectively reduced by a low soil SO_4^{2-} concentration.

The Impact of Root Size and Morphology on the Content of Sinigrin and Progoitrin of Cultivars from the Various Groups

The Brussels sprouts cultivars of table IV are categorized in 3 groups depending on their response to variations in soil SO_4^{2-} concentration and cumulative rainfall. The variable responses of cultivars to variations in soil SO_4^{2-} concentrations and rainfall might be, as stated in the previous paragraphs, related to the size and morphology of their root systems. The architecture, development and differentiation of plant root systems in general is difficult to study in soil, as removal of soil particles essentially disrupts the spatially organization of root structures and tears apart mutual connections between the tap roots, lateral roots and hairy roots. Root development was therefore studied in seedlings grown on plain water agar one week after germination.

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Water agar allows a regular root development and yields root systems which can easily be removed and evaluated for root architecture traits. We postulate that the observed root architecture of seedlings on water agar resembles the real situation in soil. Seedlings of the cultivars differed significantly in the length of the tap root, the number of lateral roots, the position of the lateral roots on the tap root, the length of individual lateral roots and the total length of lateral roots. The key root architecture traits of seedlings of the various cultivars of groups 1 to 3 are presented in table V. Surprisingly the cultivars from the 3 groups have their own characteristical root architecture. Group 1 cultivars have on average long tap roots (72.8 mm) and lateral root formation almost up to the root tip. Cultivars from group 2 have shorter tap roots (60.7 mm), V-shaped root systems and superficial lateral root formation on the top 30 mm of the tap root. Group 3 cultivars also have shorter tap roots (61.6 mm) and on average Y-shaped root systems with exclusively lateral root formation on the top 20 to 30 mm of the tap root. In a recent paper, Bushamuka and Zobel (1998) nicely described corn and soybean cultivars which expressed similar root architecture phenotypes in compacted soil layers with respect to the traits such as tap root length, number of lateral roots and length of lateral roots, as observed for Brussels sprouts on water agar. In figure 5, the root shape of group 1, 2 and 3 cultivars is depicted.

Group 3 cultivars have more superficial root systems than group 2 cultivars. The distinct root forms of the 3 groups fit with their response to variations in rainfall and soil SO_4^{2-} concentration. Group 1 cultivars do not respond to the former factors because their root system is evenly distributed in the top and subsoil layers thus warranting SO_4^{2-} uptake over the whole length of the tap root. Group 2 cultivars have a V-shaped superficial root system and are significantly dependent on the lateral roots in the top soil layer for the uptake of SO_4^{2-} The root system of group 3 cultivars is even more superficial than that of group 2 and consequently is their SO_4^{2-} uptake limited to the SO_4^{2-} availability in the top soil layer.

Table V. Linkage between root architecture traits and the sprout glucosinolate content of Brussels sprouts cultivars and their response to variations in cumulative rainfall during the production period and the soil SO_4^{2-} concentration at the start of the season. Abbreviations: sum sin + prog = sum sinigrin + progoitrin, n = number, Δsum rain = responsiveness of sum of sinigrin + progoitrin to variation in rainfall, Δsum SO_4^{2-} = responsiveness of sum of sinigrin + progoitrin to variation in soil sulfate concentration, nr = not relevant.

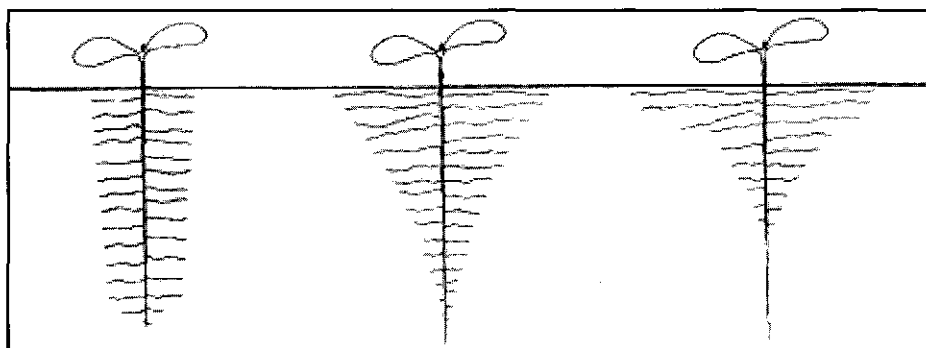| Group/description	cultivar	sum sin + prog mg/100g	lateral roots (n)	tap root length (mm)	lateral roots (cm)	Δsum rain (mg/100mm)	Δsum SO_4^{2-} (mg/1mM)	root architecture	lateral root distribution over tap root
Group 1 Rain and SO_4^{2-} insensitive	Maximus	67	16.3	83.0	12.9	nr	nr	column-shape	dispersed over 0-60 mm
	Exodus	87	9.7	70.0	7.8	nr	nr	column-shape	dispersed: 0-50 mm
	Genius	102	8.9	55.0	9.2	nr	nr	column-shape	dispersed over whole root
	SG2116	122	20.3	72.0	16.2	nr	nr	V-shape	evenly over 0-60 mm
	Stephen	184	24.0	84.0	20.3	nr	nr	V-shape	evenly over 0-60 mm
	mean group	112	15.8	72.8	13.2	nr	nr	nr	nr
Group 2 Rain and SO_4^{2-} sensitive	Angus	115	14.7	60.5	10.6	10	31	V-shape	evenly over 0-30 mm
	Origus	142	17.3	39.0	16.3	20	54	V-shape	evenly over 0-30 mm
	Ajax	153	17.7	55.5	15.1	14	37	V-shape	evenly over 0-30 mm
	Philemon	163	23.9	80.0	20.8	17	45	V-shape	evenly over 0-30 mm
	Ilias	170	23.1	68.5	29.7	60	157	V-shape	densely over 0-30 mm
	mean group	149	19.3	60.7	18.5	24	65	nr	nr
Group 3 Sensitive to an interaction between rain and SO_4^{2-}	Helemus	83	15.8	73.0	8.8	71	166	Y-shape	dispersed over 0-40 mm
	Adonis	115	16.9	51.0	11.2	72	257	Y-shape	evenly over 0-30 mm
	Louis	117	22.9	69.0	23.9	63	157	extreme Y-shape	evenly over 0-20 mm
	Tavernos	119	14.8	39.5	16.4	130	325	extreme Y-shape	evenly over 0-30 mm
	Cyrus	172	21.5	69.0	23.7	80	131	Y-shape	densely over 0-30 mm
	Lauris	196	21.8	68.2	20.9	77	187	Y-shape	densely over 0-30 mm
	mean group	134	19.0	61.6	17.5	82	204	nr	nr
	LSD	nr	2.9	11.0	3.7	nr	nr	nr	nr

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Figure 5. Shape of root systems of seedlings from group 1 (left seedling with column-shaped root system), group 2 (middle seedling with V-shaped root system) and group 3 (right seedling with Y-shaped root system) Brussels sprout F1-hybrids 1 week after germination on plain water agar.

Brussels sprouts cultivars differ significantly for the content of sinigrin and progoitrin (tables I to V). The sum of sinigrin and progoitrin of cultivars is for about 60% determined by the genotype (van Doorn et al., 1998d) and dependent on the interplay between the uptake and distribution of SO_4^{2-}, the biosynthesis rate of the glucosinolate precursor methionine and the biosynthesis rate of these glucosinolates from methionine. The significant relationships between the cumulative lateral root length of the Brussels sprout cultivars from the 3 groups and the sum of sinigrin and progoitrin are shown in figure 6. The traits are significantly correlated with square correlation coefficients between 0.74 (p = 0.06) for group 2 and 0.88 (p = 0.06) for group 1. The data of Maximus and Louis have been omitted from figure 6 since these cultivars have a much lower content of sinigrin and progoitrin as would have been expected on the basis of their cumulative lateral root length. The cumulative lateral root length of the root system is a good predictor of the sum of sinigrin and progoitrin of Brussels sprout cultivars. The deviant results for Maximus and Louis, which have a relative low content of sinigrin and progoitrin in combination with a more developed root system, seem to indicate that these cultivars either have a lower SO_4^{2-} uptake rate or a lower biosynthesis rate of glucosinolates.
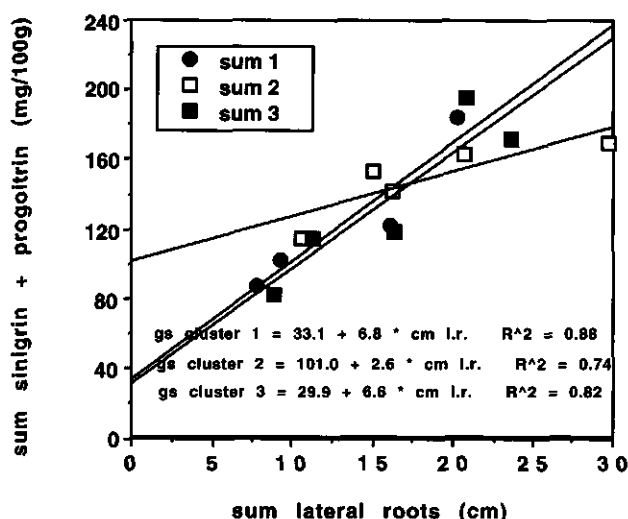
Figure 6. Relationships between the sum of lateral roots (in cm) of seedlings and the sum of sinigrin and progoitrin (in mg/100g) of Brussels sprout cultivars from groups 1 to 3. Abbreviations: l.r. = sum of lateral roots. Sum 1 to 3 denote the sum of sinigrin and progoitrin of cultivars from groups 1 to 3.

In the upper graph of figure 7 the significant relationships are shown between the cumulative sum of lateral roots and the responsiveness of group 2 cultivars to respectively variations in rainfall and soil SO_4^{2-}. The correlation coefficients observed are to some extent strengthened by the response factors of the cultivar Ilias but realistic as Ilias is an example of a very responsive cultivar with a highly developed superficial root system. The decreased content of sinigrin and progoitrin under the influence of rainfall can be attributed to the displacement of SO_4^{2-} to the subsoil layer by leaching out of the reach of the superficial lateral roots.

No relationship is observed between the tap root length of group 2 cultivars and their responsiveness to variations in rainfall and soil SO_4^{2-}. In the lowest graph of figure 7 the response of the sinigrin and progoitrin content of group 3 cultivars to interactive variations in soil SO_4^{2-} and rainfall is correlated to the seedling tap root length.

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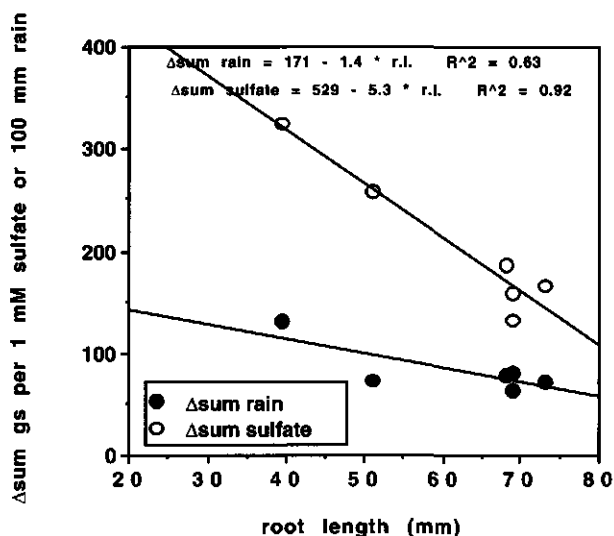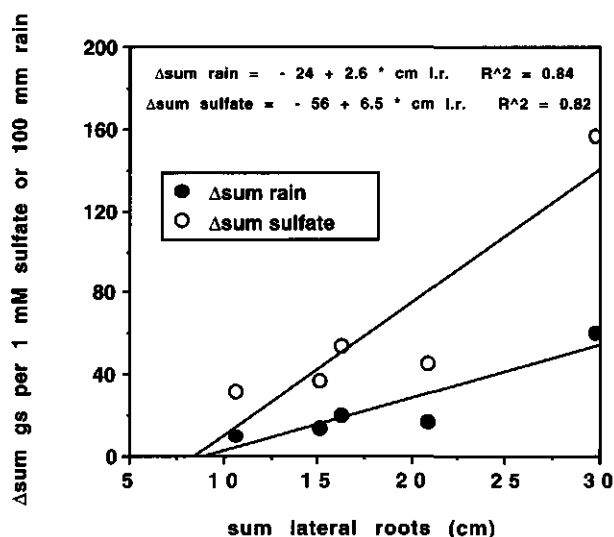**Figure 7.** Relationships between the sum of lateral roots (in cm) of group 2 cultivars (upper graph) and the tap root length (in mm) of group 3 cultivars (lowest graph) and their response to respectively variations in rainfall (Δsum rain in mg gs/100 mm rain) and soil sulphate concentration (Δsum sulphate in mg gs/mM SO_4^{2-}). Abbreviations: l.r. = sum of lateral roots, sum gs = sum of sinigrin and progoitrin.

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The variation in the sum of glucosinolates in group 3 cultivars is increasing with shorter tap root length. No relationship is observed between the cumulative lateral root length of group 3 cultivars and their responsiveness to variations in rainfall and soil SO_4^{2-}. The Y-shaped lateral root distribution pattern and the relative short tap roots are the underlying reason why group 3 cultivars have a highly variable glucosinolate content in response to variations in soil SO_4^{2-} content and rainfall. Group 3 cultivars have, due to their root architecture, a relative low evaporation-driven SO_4^{2-} uptake from sub to top layers (Marschner, 1995). The evaporation rate of the crop and the amount of rain during the cultivation period determine whether available SO_4^{2-} will reside in the top or sub soil layer during the season. The results of table V and figures 6 and 7 clearly demonstrate that root architecture traits of one week old seedlings can be used to predict the content and stability of sinigrin and progoitrin of cultivars from the 3 different response groups to soil SO_4^{2-} content and rainfall.

Sulfate Uptake of Young Plants of Brussels Sprouts Cultivars on a Hydroponic System

The content of glucosinolates of cultivars is determined mainly by the density and distribution of the root system in the soil and the SO_4^{2-} concentration in its vicinity. These findings suggest that the specific SO_4^{2-} uptake capacity of root systems of Brussels sprout cultivars, defined as the SO_4^{2-} uptake per unit root surface area or plant weight, is of minor importance and apparently not the limiting factor in our experiments. Plants have a demand-driven SO_4^{2-} uptake which is upregulated upon SO_4^{2-} starvation (Lappartient and Touraine, 1996). In order to prove our hypothesis SO_4^{2-} uptake experiments were conducted with the Brussels sprout cultivars of table III and V using young plants which had previously been raised using a hydroponic system with nutrient solutions with 1.6 mM SO_4^{2-}, representing the SO_4^{2-} concentration at the start of the season, and 0.2 mM SO_4^{2-} as a limiting concentration observed in low SO_4^{2-} soils and during the sprout formation period of cultivars.

In table VI the average "five-seedlings" weight for the cultivars of the three groups on nutrient solution with respectively 0.2 and 1.6 mM SO_4^{2-} and their mean daily

consumption of nutrient solution during the uptake experiment are presented, both attributes which might be relevant for the SO_4^{2-} uptake rate. A concentration of 0.2 mM SO_4^{2-} in the nutrient solution did not have any negative influence on the growth and development of young plants of cultivars from the 3 groups in comparison to 1.6 mM SO_4^{2-} The seedlings raised on 0.2 mM even tended to have a higher weight and produced leaves with a dark-green color. The plant weight of cultivars from the three groups did not differ between SO_4^{2-} concentrations. The average daily consumption of nutrient solution during the SO_4^{2-} uptake experiment was comparable between groups and not affected by the SO_4^{2-} concentration. The average SO_4^{2-} uptake rate of the cultivars from the three groups was relatively low when raised on 1.6 mM SO_4^{2-} and increased more than threefold when grown on 0.2 mM SO_4^{2-}, demonstrating the stimulatory effect of SO_4^{2-} limitation on the SO_4^{2-} uptake rate of plants. The SO_4^{2-} uptake rate of cultivars was highly variable between the cultivars of the corresponding groups and neither related to any of the root architecture traits nor to their sum of sinigrin and progoitrin (data not shown). The SO_4^{2-} uptake rate of young Brussels sprouts plants was translated to the field situation by means of linear extrapolation. In the far right column of table VI it is shown that the cultivars of the three groups with the lowest SO_4^{2-} uptake capacity, when grown on a 0.2 mM SO_4^{2-} containing nutrient solution, still can take up more than 2 kg S d^{-1} ha^{-1} after a period of SO_4^{2-} deprivation. The highest S uptake capacity observed for any cultivar was 15 kg S d^{-1} ha^{-1}.

Our measured SO_4^{2-} uptake rate maximum is lower than the extrapolated maximum uptake of 25 kg S d^{-1} ha^{-1} observed for the *Brassica* canola by Lappartient and Touraine (1996). Their SO_4^{2-} uptake rates vary between approximately 6 and 12 kg S d^{-1} ha^{-1} after short periods of SO_4^{2-} deprivation, conditions which resemble the concentration of 0.2 mM in our experiments, are however in line with those of Brussels sprout cultivars.

The SO_4^{2-} uptake experiments show that the SO_4^{2-} uptake rate of Brussels sprout cultivars is not a limiting factor in SO_4^{2-} fed root systems, as even the cultivars with the lowest SO_4^{2-} uptake

Table VI. Average SO_4^{2-} uptake rate and related attributes of young plants of cultivars from the three response groups of Table 4 after growth on a hydroponic system with nutrient solutions containing 0.2 mM (limiting) and 1.6 mM (optimal) SO_4^{2-}. The values of the attributes in the table are expressed per five young plants. The standard deviation of the mean for the treatments is presented between brackets. The S-uptake capacity of young plants of cultivars is linearly extrapolated to the field situation assuming a cumulative plant weight of 73 ton per hectare. The groups did not differ significantly for the traits evaluated (analysis of variance at p = 0.05).

group	plant weight g 5 plants⁻¹		uptake nutrient solution mL d⁻¹ 5 plants⁻¹		SO_4^{2-} uptake rate µmol S d⁻¹ 5 plants⁻¹		sulfur uptake capacity of cultivars kg S d⁻¹ ha⁻¹ (on 0.2 mM)	
	1.6 mM	0.2 mM	1.6 mM	0.2 mM	1.6 mM	0.2 mM	lowest cultivar	highest cultivar
1	9.1(1.7)	10.1(1.8)	17.7(2.5)	20.8(3.0)	5.2(6.7)	18.8(21.8)	2.1	10.6
2	9.7(1.7)	10.3(1.9)	20.3(2.7)	21.0(3.0)	9.4(13.0)	33.7(31.9)	2.6	15.0
3	11.3(1.8)	11.4(2.6)	20.2(2.4)	20.3(3.5)	8.4(11.3)	30.8(30.8)	2.2	8.1

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can take up far more SO_4^{2-} than the quantities normally found in soils. The SO_4^{2-} uptake of roots in a hydroponic system is, unlike in soils, not limited by mass flow of SO_4^{2-}. The suppression of SO_4^{2-} uptake in root systems of Brussels sprouts cultivars at higher SO_4^{2-} concentrations is probably the reason why substantial SO_4^{2-} is transported to sub soil layers during rainfall when group 2 and 3 cultivars with superficial root systems are grown. SO_4^{2-} leaching to sub soil layers by rain will predominantly occur in the first period after planting when the soil SO_4^{2-} concentrations are between 0.8 mM (shortly after planting) and 0.4 mM (two months later) and are high enough to suppress SO_4^{2-} uptake.

Production of Brussels Sprouts with an Acceptable Glucosinolate Content for Consumers: Relevant Factors for Growers

1. Soil SO_4^{2-} Concentration

The production of a high yield of Brussels sprouts with an attractive appearance and acceptable glucosinolate content for consumers is practically possible but demands some extra measures from professional growers. The results of this paper show that the environmental influence on the sinigrin and progoitrin of varieties is mainly determined by the soil SO_4^{2-} concentration at the start of the production period and the amount of rain during the production period. Based on the results of our experiments we advise growers to determine the soil SO_4^{2-} concentration at the production site prior to fertilizer application and planting when they plan to grow cultivars whose glucosinolate accumulation is stimulated by a high soil SO_4^{2-} concentration. The variation in soil SO_4^{2-} concentration between 0.2 (Staden, Belgium, table I) and 1.8 mM (Groningen, table II) in this paper, values which are in line with those reported by Nuttall and Ukrainetz (1991), demonstrate a large variability in soil SO_4^{2-} concentrations between different sites and soil types. The soil SO_4^{2-} concentration has to be the basis for the quantity of sulfur which should be included in the fertilizer applied. The soil SO_4^{2-} concentration after fertilization has to be such that it

prevents the accumulation of more than 2.2 g kg^{-1} sinigrin and progoitrin, the critical level for the sum of these glucosinolates for consumers (van Doorn et al., 1998a). Based on the results of this paper a soil SO_4^{2-} concentration of 0.5 mM SO_4^{2-} at the start of the growth period will guarantee an acceptable sum of sinigrin and progoitrin in varieties which have a SO_4^{2-}-sensitive glucosinolate accumulation.

2. S-Demand of the Crop

A soil SO_4^{2-} concentration of 0.5 mM corresponds to approximately 50 kg S ha^{-1} in the 0 to 30 cm layer which is much lower than the S-demand of respectively 100 and 300 kg S ha^{-1} for Brassica crops reported by Wildeman (1997) and Dijksterhuis and Oenema (1990). Fifty kg S ha^{-1} did not lead to sulfur deficiency symptoms and yield loss for the varieties in the various field trials of this paper (data not shown) suggesting that Brussels sprout varieties perform well at relatively low soil SO_4^{2-} levels. Our findings are in contrast with the results of Wildeman (1997) who reported stagnation of growth of Brussels sprouts due to SO_4^{2-} deficiency in the soil. The observed problems by Wildeman were however related to the quality of the fertilizer applied and almost certainly to a low soil SO_4^{2-} concentration at the start of the growth period. Apparently the soil was already deficient in SO_4^{2-} before planting and, moreover, dressed with SO_4^{2-}-free fertilizer.

SO_4^{2-} deficiency symptoms and yield loss are observed when the content of essential proteins and enzymes decreases below a critical level after a severe shortage in SO_4^{2-} (Stuiver et al., 1997). Sulfur deficiency symptoms were observed in double low rapeseed varieties (containing low seed glucosinolate and erucic acid levels) below 0.35% (w/w) sulfur or 3.5 g S kg^{-1} dry matter (Schnug and Haneklaus, 1993). Sulfur deficiency symptoms can also be triggered by shortage in iron as sulfur and iron together form essential iron-sulfur clusters for key enzymes in several metabolic pathways (Imsande, 1998). Recently comparable and high total S levels above 0.5% (w/w) dry matter were measured in healthy leaves and leaves with, according to experts, heavy S-deficiency symptoms, demonstrating that S-deficiency disorders are not necessarily correlated to SO_4^{2-} limitations (personal

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communications Mr Moraal, crop consultancy ALF BV, Dronten, The Netherlands). The distribution of sulfur between proteins, secondary metabolites and inorganic SO_4^{2-} has been measured in various *Brassicas*. In rapeseed the hydrochloric acid digestible S-content, which is mainly protein-derived, is close to 3 g S kg^{-1} dry matter and almost constant at S-applications between 20 and 80 kg S ha^{-1} (Withers and O'Donnell, 1994). In the same study, the water soluble inorganic SO_4^{2-} content of rapeseed, including SO_4^{2-} originating from glucosinolates due to the way of sample preparation, was highly variable in different environments. Comparable total S-content values have been observed in canola varieties (Nuttall and Ukrainetz, 1991). These results indicate that *Brassicas* need a specific amount of sulfur for the synthesis and maintenance of structural proteins and enzymes and start to accumulate sulfur containing secondary metabolites when this demand has been met. In Brussels sprouts a protein-derived S-content of 3 g S kg^{-1} dry matter is equivalent to 33.8 kg S ha^{-1} and the application of 50 kg S ha^{-1} is therefore more than sufficient to meet their S-demand. The recycling of SO_4^{2-} from abscised leaves during the sprout production period (figure 2) ensures the supply of sulfur at a later stage of crop development.

3. The Choice of Cultivars

Brussels sprout cultivars differ significantly for the content of sinigrin and progoitrin and in their responsiveness to variation in soil SO_4^{2-} concentration and rainfall. Cultivars with a low sum of glucosinolates such as Maximus, Exodus and Genius produce tasty sprouts in a wide range of environments. Other cultivars will produce tasty sprouts only when grown on soils with a SO_4^{2-} content not higher than 0.5 mM.

4. Interactions between Genotype and Environment

Crucifers such as *Brassica nigra* and rapeseed start to accumulate glucosinolates at higher SO_4^{2-} levels (Marquard et al., 1968; Schnug and Haneklaus, 1993). The Brussels sprout cultivars currently available on the market can contain up to 500 mg $100g^{-1}$ fresh matter glucosinolates (= 50 kg S ha^{-1}) (van Doorn et al., 1998d) and up to 30 mM S-methyl-cysteine sulfoxide (SMCO) (= 60 kg S ha^{-1}), a sulfur containing amino acid which accumulates at high levels during the winter (personal observations, data not

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shown). These figures demonstrate that specific Brussels sprout cultivars can accumulate up to 140 kg S ha^{-1} at saturating S-fertilization, distributed for approximately 20% : 80% over proteins and secondary metabolites respectively.

The linear relationships observed between the soil SO_4^{2-} concentration and the sum of sinigrin and progoitrin of many cultivars show that the accumulation of secondary metabolites such as glucosinolates in Brussels sprouts is strictly dependent on the availability of SO_4^{2-}. The relationships between the soil SO_4^{2-} concentration and the sum of sinigrin and progoitrin of cultivars were observed at various production sites (tables I and II) and production seasons (tables III and IV). "Luxury" consumption of SO_4^{2-} by Brussels sprout cultivars stimulated the accumulation of sinigrin and progoitrin levels above 200 mg 100 g^{-1} in cultivars such as Ilias, Cyrus and Lauris. Such levels are unacceptable for consumers (van Doorn et al., 1998a).

5. Rainfall during the Growth Period.

Rainfall plays a dual role in the SO_4^{2-} supply of Brussels sprouts. Rain is responsible for the liberation of mineralized SO_4^{2-} from abscised leaves and for the transfer of SO_4^{2-} from the top soil layer to sub soil layers out of the reach of superficial root systems. The latter role seem to be most relevant as low rainfall during the growth period stimulates the accumulation of glucosinolates in Brussels sprout cultivars. SO_4^{2-} present in the sub soil layer is not lost for the crop. SO_4^{2-} is leached to deeper soil layers by rain but transported back to the top layer by evaporation driven mass flow (Marschner, 1995). A Brussels sprout crop can evaporate up to 8.10^5 L ha^{-1} water per day during the phase of maximal growth (van Pol, 1984), a volume which is sufficient to mediate the mass flow of SO_4^{2-} from the sub soil layers back into the reach of the root system of the crop.

The cumulative rainfall during the production period of the cultivars in table III varied between 424 and 717 mm rain which, according to Dijksterhuis and Oenema (1990), corresponds to a decrease in the soil SO_4^{2-} concentration in the 0 to 30 cm layer of 2.2 and 3.8 mM SO_4^{2-} respectively. The average decrease in the sum of sinigrin and progoitrin of the cultivars of groups 2 and 3 is 24 and 79 mg 100 mm^{-1} rain respectively (table IV). The

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maximum difference in rainfall of 293 mm between years corresponds on average with 70 mg and 230 mg 100 g^{-1} sinigrin and progoitrin for the cultivars of group 2 and 3 respectively. These quantities of glucosinolates are equivalent to 7.2 and 23.5 kg S ha^{-1} and a concentration of 0.07 and 0.24 mM soil SO_4^{2-} respectively. The calculations clearly show that the decrease in soil SO_4^{2-} concentration, as derived from the variation in the sinigrin and progoitrin content, is only a fraction of the predicted value. The water uptake of the crop during growth seem to be the underlying reason why the decrease in the soil SO_4^{2-} concentration is smaller than the value obtained by extrapolation from rainfall figures.

6. The Root Architecture of Cultivars

Our experiments have shown that the root shape and the cumulative lateral root length of cultivars mainly determine how much of the soil SO_4^{2-} will be taken up by the root system of the crop. The variation in root shape between the various Brussels sprout cultivars is not related to their performance in terms of vigor and yield. The continuous selection for Brussels sprout cultivars with a high quality on well-fertilized soils has apparently resulted in productive cultivars which rely on the uptake of nutrients in the topsoil layer. The SO_4^{2-} uptake experiments with the cultivars from the three groups in table VI also demonstrate that the SO_4^{2-} uptake capacity of Brussels sprout cultivars is neither related to the sum of sinigrin and progoitrin nor to the variability of these glucosinolates under the influence of environmental conditions. The high SO_4^{2-} uptake capacity of cultivars and the upregulation of SO_4^{2-} uptake at low soil SO_4^{2-} concentrations are favorable traits for precision dressings of sulfur containing fertilizer during the season.

7. The Consumer and Retailers

The majority of the consumers in NW-Europe have been informed by retailers about the availability of sprouts with a good flavor by means of commercials and product labeling. Professional sprout growers should supply consumers with sprouts which meet their expectations in terms of appearance and taste to maintain their market share. A constant supply of tasty high quality sprouts should be based on the choice of cultivars

and balanced fertilizer management with respect to SO_4^{2-}. Sprouts with a low sinigrin and progoitrin content can be obtained by cultivar choice (e.g. Maximus and Exodus) and by the right fertilizer management. Soil samples should be used to screen the soil SO_4^{2-} concentration of sprout production sites to avoid the application of SO_4^{2-} above a critical level. Growers who apply fixed high amounts of up to 80 kg S ha^{-1} to avoid yield loss and sulfur deficiency symptoms, as advised by some consultants, risk producing sprouts with a bitter taste. It is better for growers to start the production of sprouts with a soil SO_4^{2-} concentration of 50 kg S ha^{-1} and apply small amounts of SO_4^{2-} fertilizer when excessive rainfall demands such measures, as they already do for N-dressings. With careful SO_4^{2-} management, growers can produce good tasting sprouts with cultivars such as Lauris and Ajax which are normally considered to be examples of bitter varieties.

Classic breeding for a low sinigrin and progoitrin on the one hand (van Doorn et al., 1998d) and a proper SO_4^{2-} management on the other hand are powerful tools for the production of tasty sprouts during the whole season.

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Chapter 7
Hormonal and physiological characterization of an ethylene insensitive long shelf life Brussels sprout inbred line with a high cytokinin content and a low polar auxin transport capacity.

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Abstract

Various unique hormone responses are described of a Brussels sprout inbred line which produces long shelf life (LSL) sprouts. Seedlings from the inbred line display a normal triple response to ethylene but detached leaves and sprouts, unlike those of other inbred lines, are insensitive to high doses of ethylene, the auxin 2,4-D and the polar auxin transport inhibitor 9-hydroxy-fluorone-carboxylic acid (HFCA). Sprouts from the inbred line and derived cultivars have a high cytokinin content. The sprouts have a basal ethylene production which cannot be stimulated by externally added ethylene. This inbred line demonstrates that the expression of an LSL phenotype in sprouts, and presumably other edible plant parts, is under complex hormonal control and is stimulated by an interaction between a high cytokinin content and a low rate of polar auxin transport.

Introduction

Fruits and vegetables comprise an essential part of human diet and form the basis for the intake of essential nutrients such as vitamins, minerals and dietary fibres. Selection and purchasing of specific fruits and vegetables by consumers is, nevertheless, mainly based on visible quality criteria such as fresh appearance and the absence of cosmetic defects. Fruits and vegetables in general can only be stored for a limited period after harvest and their initial freshness quickly deteriorates due to senescence driven processes. The post harvest deterioration of fruits and vegetables can, depending on the crop, be based on dehydration (lettuce), softening (tomatoes), rotting (cherries), yellowing of wrapper leaves (cabbage, Brussels sprouts) or a combination of these processes. Most of these shelf life limiting processes are controlled by the hormone ethylene (Yang and Hoffman, 1984). For these reasons, the introduction of a long shelf life of produce has a high priority in the breeding programs of almost all fruit and vegetable crops. The shelf life of fruits and vegetables can be defined as the post harvest period in which the product appears fresh and attractive to consumers.

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Currently, extension of the shelf life of fruits and vegetables is based on various approaches which can roughly be subdivided into 3 groups in descending order of importance: -1- modification of the storage atmosphere, -2- breeding using spontaneous mutants with a longer shelf life and -3- genetic engineering e.g. down regulation of ethylene production or perception genes. A storage environment with low O_2 and raised CO_2 concentrations and a low temperature (Controlled Atmosphere (CA) storage) prevents the ethylene stimulated yellowing of white cabbage (Hicks and Ludford, 1980; Hicks et al., 1982) and Brussels sprouts (Lipton and Mackey, 1987). The shelf life of tomatoes can be extended by the introduction of the recessive rin (ripening inhibitor) and nor (non-ripening) genes which favour firmness by decreasing the tomato softening rate (Kopeliovitch et al., 1979). During the last decade, genetic engineering has been applied to the improvement of shelf life in tomatoes. Suppression of the activity of the enzymes ACC synthase (Oeller et al., 1991) and ACC oxidase (Gray et al., 1994), which, respectively, catalyze the biosynthesis of ACC and ethylene have been reported to improve tomato shelf life. Degradation of the ethylene precursors ACC and SAM by expression of, respectively, ACC deaminase (Klee, 1993) and SAM hydrolase (Kramer et al., 1996) has the same effect. Transgenic tomato varieties with modified ethylene responses and extended shelf life based on the former principles are close to the market.

The shelf life of vegetables is clearly limited by the production of and sensitivity to ethylene. This phytohormone initiates many aspects of fruit ripening and senescence related processes in edible plant tissues (Yang and Hoffman, 1984). The design of the ethylene biosynthesis route, starting with the amino acid methionine, the perception of ethylene by specific receptors and the composition of the signal transduction pathways leading to specific ethylene responses, including senescence, have recently been reviewed (Bleecker and Schaller, 1996; Lelievre et al., 1997; Kieber, 1997) with specific emphasis on tomato (*Lycopersicon esculentum*) and the weed *Arabidopsis thaliana*. The senescence and abscission process of intact leaves is preceded by a climacteric rise in ethylene production (Morgan et al., 1992).

The production of ethylene by plant parts and their sensitivity to the compound are for a significant part determined by interactions with other phytohormomes such as cytokinins and auxins (Klee and Romano, 1994; Barendse and Peeters, 1995). The expression of ethylene biosynthesis genes is stimulated by auxins (Abel et al., 1995) and cytokinins (Vogel et al., 1998a and b). Ethylene production of plants also is stimulated by both auxins (Mito et al., 1995; Sabba et al., 1998) and cytokinins (Vogel et al., 1998a and b). Cytokinins in general delay the senescence process in detached and attached plant parts. For instance, broccoli florets treated with cytokinin display extended shelf life due to a reduced respiration rate and delayed chlorophyll degradation (Downs et al., 1997; Rushing, 1990). The fact that cytokinins suppress leaf senescence was nicely demonstrated by Gan and Amasino (1995) in transgenic tobacco expressing a cytokinin biosynthesis IPT gene under the control of a senescence-driven promoter. Auxins generally promote the senescence in plant parts. In squash, auxin stimulates the production of ethylene via a specific auxin sensitive ACC synthase (Takahashi et al., 1995) while inhibition of polar auxin transport promotes ethylene mediated flower abortion in pepper plants (Huberman et al., 1997). These examples suggest that ethylene production and ethylene triggered senescence processes of plant parts are controlled by their cytokinin-auxin ratio (CAR) in the physiological concentration range of both hormones. A high CAR seems to be associated with down regulation of ethylene production, a lack of ethylene perception resulting in a longer shelf life. A low CAR is correlated with a high ethylene production, ethylene perception and senescence related processes. The mutual interactions between cytokinin and auxin metabolism (both groups of hormones stimulate each others conjugation and oxidation, Eklof et al., 1997; Zhang et al., 1995; Crouch and van Staden, 1995), further underline the complexity of the control of ethylene production in plants.

Ethylene only triggers senescence in plant parts which are competent to respond (Klee and Romano, 1994). Senescence of plant parts is dependent on age (e.g. Oh et al., 1997). This statement supports the observation that the oldest leaves from

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plants of most crops usually turn yellow and abscise first. The ripening of tomato clusters begins with the proximal (oldest) fruits. The life span of plant parts and their capacity to resist ethylene depend for a significant part on their sink strength, the capacity to import or produce assimilates, hormones and nutrients for growth and maintenance. According to Bangerth (1989) the sink strength of plant organs and their dominance over other parts is dependent on their auxin production and polar transport capacity. The sink strength of leaves is, however, also dependent on cytokinin activity as benzyladenine treated leaves from pumpkins and broad beans shift from export (source mode) to accumulation (sink mode) of sugars (Ron'zhina et al., 1995). Eklof et al. (1997) found the IAA content of the youngest leaves of tobacco plants, (a model plant with respect to age-dependent leaf senescence), to be tenfold higher than the content of the senescence sensitive oldest leaves which is in line with the hierarchy of leaves on the plant. The observation by Li et al. (1995) that cytokinins antagonize the expression of polar auxin transport mediated apical dominance in peas further demonstrates that auxins and cytokinins are the key hormones in determining whether plant organs contribute to growth and development or become senescent.

Many leafy vegetable crops deteriorate quickly after harvesting even during storage under shelf life promoting conditions such as low temperature, ethylene scavenging, elevated CO_2 concentration and controlled humidity. The shelf life of leafy Brassica vegetables in particular is, except for red and white cabbage, very short and for crops such as broccoli and Brussels sprouts less than one week due to a high level of ethylene production (Tian et al., 1994). Brussels sprouts is a local crop which is typically produced and consumed in NW European countries, Australia and some states in the USA. Introduction of shelf life into Brussels sprout varieties will considerably increase their sales potential. Extension of the shelf life of sprouts by storage under low oxygen concentrations is not advisable as such conditions might trigger the production of sulphur-containing off-flavours (Marks et al., 1992; Lipton and Mackey, 1987). Genetic engineering of shelf life in a crop such as

Brussels sprouts is not economically feasible as the costs of such a process and the possible sales of the modified varieties are out of proportion. The introduction of longer shelf life in the Brussels sprout germplasm will therefore be dependent on the occurrence of natural mutations in the germplasm and the subsequent recognition of shelf life traits by effective screenings.

In this paper a Brussels sprout inbred line with extremely long shelf life is presented and characterized in terms of hormonal and physiological responses. Sprouts from the inbred line remain fresh and green for three weeks in the presence of up to 50 ppm ethylene in the storage atmosphere. Seedlings expressing the LSL trait can be selected by their capacity to germinate in the presence of cytokinin conjugation inhibitors and their low level of polar auxin transport. The LSL trait discovered confers ethylene insensitivity to sprouts and leaves from the plants and is modulated by a concerted action between polar auxin transport and cytokinin metabolism.

Results

Strategy for the Creation and Selection of a LSL Brussels Sprout Inbred Line

Most commercial varieties of the various Brassica crops, such as Brussels sprouts and broccoli are F1-hybrids, the result of a cross between a female and male parental inbred line, both expressing a unique set of homozygous traits. In classical F1-hybrid breeding, the development of parental inbred lines is generally started by self pollination of interesting F1-hybrids or the crossing of phenotypically desirable plants. Repeated self pollinations, and the selection of phenotypically attractive plants in the segregating populations during every inbred cycle finally results in new parental inbred lines which combine a new set of interesting traits, which are the genitors for new varieties.

The development of Brussels sprout parental inbred lines with extended shelf life was initiated by the selection of plants whose sprouts maintained a fresh and green appearance in the field until March, 11 months after planting. The standing ability of Brussels sprout varieties and inbred lines in general rarely exceeds 8 to 9

months, as buttons thereafter start to senesce and deteriorate quickly on the plant. Essentially, all plants of interest had to combine the standing ability trait with lateness and an optimal button quality in terms of shape and size. The shelf life genitors were crossed to yield a collection of F1 hybrids for the development of parental inbred lines with extended shelf life according to the method in the previous paragraph.

"Standing ability" in sprout plants, means that button quality on the plant is maintained; this is not synonymous with post harvest LSL of buttons, but all plants expressing LSL will display standing ability. Harvested sprout buttons of the current commercial varieties senesce in a climacteric way and turn yellow in about 5 days after storage at an ambient temperature of approximately 20 °C. Plants with putative expression of LSL were selected when their sprouts could maintain their freshness for a post harvest period of 14 days after a senescence promoting treatment. Senescence of buttons was promoted by spraying with 100 mg/L Ethrel, a labile ethylene precursor, which resulted in complete deterioration of the sprouts of classical varieties lacking shelf life within one week. During the inbred cycles for selection of extended shelf life, a gradual but rather slow increase in shelf life was observed for individual plants of specific inbred lines. In inbred line TC130 (an in-house breeding code) the desired LSL phenotype was observed after 4 inbred cycles with selection pressure for LSL. The hormonal and physiological characterization of plant TC130-4-3-4-1 will be presented in the following part of this paper.

TC130 Seedlings Display a Normal Triple Response in the Presence of Ethylene.

The inbred line TC130 was selected by means of a screening using Ethrel as previously described. The ethylene insensitivity of TC130 sprouts might be due to a mutation in the ethylene receptor ETR (Bleecker and Schaller, 1996) or an ein-like mutation in the ethylene signal transduction pathway (Ecker, 1995).

Ethylene sensitivity of plants is classically screened by means of the induction of the triple response by ethylene in etiolating seedlings in the dark (Kieber, 1997). Developing seedlings exposed

to ethylene display short hairy radicles, short thick hypocotyls and exaggerated apical hooks. Seedlings of the LSL inbred line TC130 and the classical inbred line TC25 both display a dose dependent ethylene sensitivity when exposed to a concentration range of 0-15 mg/L Ethrel during germination on paper.
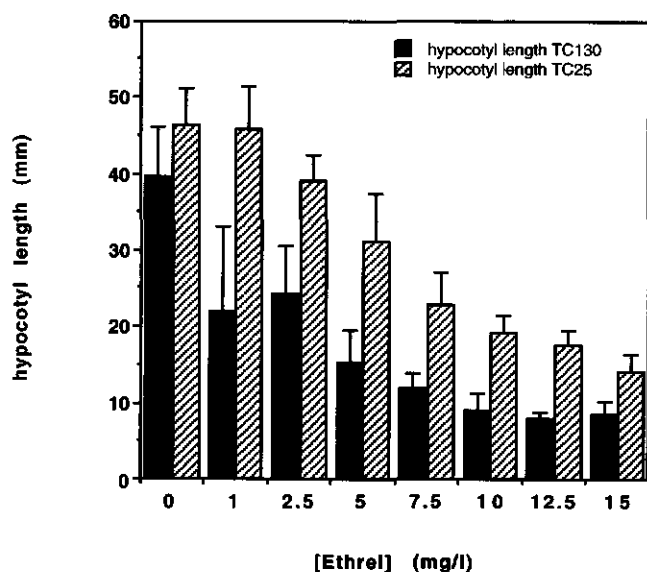

Figure 1. Inhibition of hypocotyl elongation of TC130 (LSL) and TC25 (non-LSL) seedlings as function of the Ethrel concentration. Seeds of both lines were germinated on paper saturated with the Ethrel concentration range of 0 to 15 ppm and evaluated for hypocotyl length 7 days after sowing.

In figure 1 the hypocotyl length, one component of the triple response, of TC130 and TC25 seedlings is presented as a function of the Ethrel concentration. Both inbred lines display a classical dose-dependent inhibition of hypocotyl elongation in the presence of ethylene. Seedlings from both inbred lines also displayed exaggerated apical hook formation and short hairy roots at higher Ethrel concentrations (data not shown). The relative hypocotyl elongation of TC130 seedlings is even more inhibited at increasing Ethrel concentrations than that of TC25. The hypocotyl length of the classical inbred line is reduced to 33% with 15 mg/L Ethrel compared to the initial length with water. The hypocotyl

length of TC130 seedlings is reduced to 23% under these conditions. The expression of LSL in the sprouts of inbred line TC130 apparently is not correlated with the lack of the ethylene mediated triple response in seedlings, suggesting that neither the ethylene receptors nor specific components of the ethylene signal transduction pathway are involved in the expression of shelf life in inbred line TC130.

Dose Dependency of the Induction of Senescence in Sprout Buttons by Ethrel Sprays.

Sprout buttons of classical cultivars have a short post harvest shelf life as their wrapper leaves in general turn yellow in about 5 days after picking. The shelf life of the inbred line TC130 was determined after a spray with 100 mg/L Ethrel which might not be the optimal concentration for the induction of senescence. A possible dose-dependency of senescence induction by Ethrel was tested on sprouts from the classical variety Corinth and the inbred line TC130 respectively. In figure 2 the influence is shown of Ethrel concentrations in the spray between 0 and 200 mg/L on the rate of yellowing of Corinth and TC130 sprouts during one week after spraying. The usually 6 wrapper leaves of buttons of TC130 sprouts remained green at all Ethrel concentrations during the evaluation period of 1 week, showing that TC130 sprouts are essentially insensitive to a broad concentration range of Ethrel. Buttons from Corinth quickly and dose-dependently turned yellow after the treatments. The optimal dose for the stimulation of senescence in sprout buttons is close to 100 mg/L, as a dose of 200 mg/L hardly increased the rate of yellowing.

The lack of response of TC130 buttons to Ethrel sprays might be due to a genotype specific slow release of ethylene during the experiment. Figure 3 presents the headspace ethylene concentration 2 days after sprays with water or 200 mg/L Ethrel and the number of yellow leaves on sprouts of 7 different cultivars 7 days after spraying.

Sprouts from inbred line TC130 and Spr 110, a TC130 derived F1-hybrid, did not turn yellow at a headspace ethylene concentration of respectively 91 and 64 μl L^{-1} g^{-1} while sprouts from Corinth and Philemon had turned yellow at much lower concentrations.
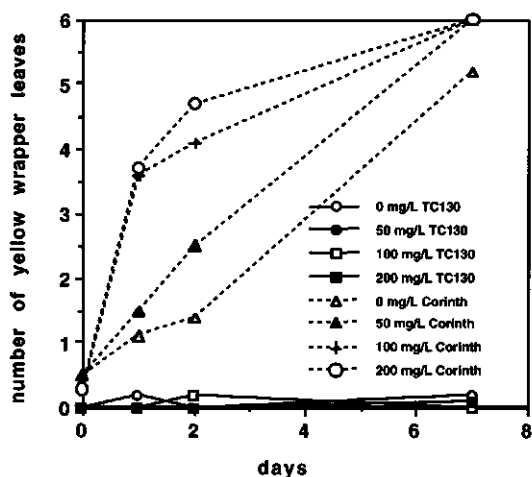
Figure 2. Dose-dependent stimulation of yellowing of sprout wrapper leaves from the LSL inbred line TC130 and the cultivar Corinth in time. Sprouts from TC130 and Corinth were sprayed with 0, 50, 100 and 200 mg/L Ethrel and evaluated for the development of yellow wrapper leaves during the following seven days. The number of yellow leaves on sprouts of both genotypes on any day is expressed as the mean of 10 sprouts for all treatments.
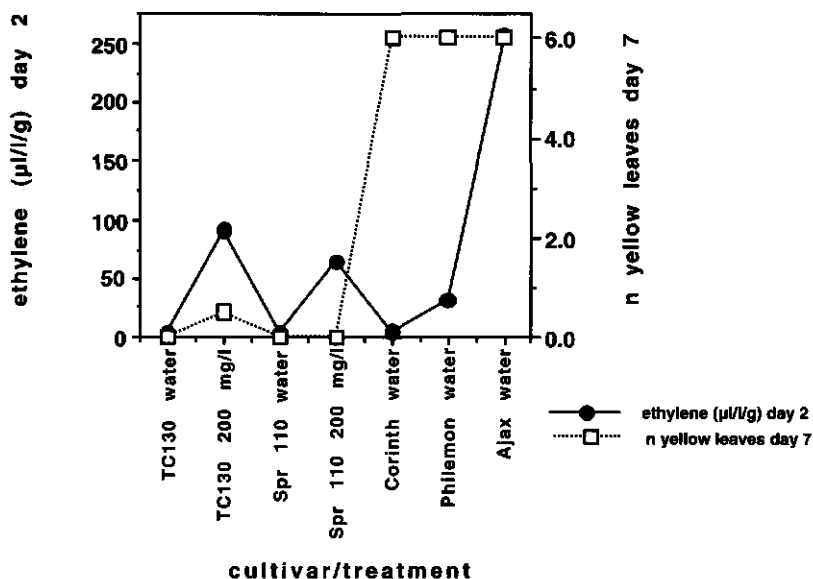

Figure 3. The ethylene production of sprouts (left vertical axis) and the number of yellow leaves on sprouts (right vertical axis) from various cultivars after sprays with water or 200 mg/L Ethrel. The lines through the data points are presented for graphical purposes only. 200 mg/L denotes 200 mg/L Ethrel.

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The high ethylene production of Ajax sprouts seem to be linked to their overmaturity, as Ajax is an earlier cultivar than Corinth and Philemon. The results show that ethylene is formed in the headspace above sprouts from all inbred lines and cultivars, regardless of the genotype. Sprouts from TC130 and derived cultivars are apparently ethylene insensitive as even at high ethylene concentrations hardly any senescence is observed.

The Senescence Process in LSL and Classical Sprouts after Ethrel treatments.

The senescence process in sprout buttons from various Brussels sprout varieties typically proceeds in different ways. Deterioration of buttons is mainly observed as yellowing of the wrapper leaves, but also other aspects such as drying, rotting and occasionally a combination of these parameters can be observed. Some senescence types are demonstrated in figure 4 where the senescence process in sprouts from the LSL inbred lines TC130 and TT29 (inbred line derived from TC130) and the classical inbred lines TC59, TC25, DC69 and TC55 is followed for 2 weeks after a spray with water or 100 mg/L Ethrel. Figure 4 and its legend are presented on respectively page 194 and 195 for print-technical reasons. In figures 4A to 4F freshly harvested buttons of the inbred lines are presented in detail before the treatment with water or Ethrel (left side photographs from top to bottom). Sprouts from both LSL inbred lines and the four classical inbred lines have on average the same quality in terms of fresh green colour and size.

In figures 4G (right side, top photograph) and 4H (right side, middle photograph) sprouts of the inbred lines are shown one week after a spray with water (bottom boxes) or 100 mg/L Ethrel (top boxes). In figure 4G the sprouts from inbred lines TC130 and TT29, as present in the left two pairs of boxes, are compared with classical sprouts from the inbred lines TC59 and TC55 respectively in the right two pairs of boxes. Sprouts of the LSL inbred lines TC130 and TT29 are not affected by neither treatment and still have a fresh and green appearance. Sprouts from TC59 and TC55 deteriorated due to a soft rot. In figure 4H the same LSL inbred lines are compared with sprouts from the

inbred lines TC25 and DC69 after comparable treatments. Sprouts from the inbred line TC25 turned yellow, sprouts from inbred line DC69 also suffered from soft rot.

In figure 4I (right side, bottom photograph) sprouts of the LSL inbred lines TC130 and TT29 and the classical inbred line TC25 are presented two weeks after the treatments. The inbred lines TC59, TC55 and DC69 were totally rotten shortly after the first evaluation and were removed from the experiment. The buttons of both LSL inbred lines were still green and fresh after two weeks while the buttons from inbred line TC25 were totally senesced. Sprouts from the inbred lines TC130 and TT29 could obviously maintain a green and fresh appearance for two weeks, even after a treatment with 100 mg/L Ethrel which was deleterious to classical sprouts.

LSL Sprouts have a Lower Ethylene Production than Classical Sprouts.

The shelf life of fruits and vegetables is often inversely related to the autocatalytic production of ethylene (Yang and Hoffman, 1984). Sprouts from classical Brussels sprouts cultivars have a short post harvest shelf life, comparable to that of broccoli, a high ethylene producer (Tian et al., 1994). The relationship between the post harvest shelf life of sprouts from the TC130 inbred line and a derived F1-hybrid (Spr 110) and 3 classical F1-hybrids (Philemon, Corinth and Kundry) and their ethylene production rate was studied in closed glass jars. In practice a lag phase of a couple of days is observed between an ethylene treatment and the induction of senescence in Brussels sprouts (see figure 2).

For this reason the initial ethylene production of buttons of the cultivars during the first 3 days was compared with the post harvest yellowing rate 6 and 14 days after the start of the experiment. The ethylene concentration in the headspace of the jars, normalized for sprout mass and jar volume, steadily increased with time in a classical cultivar like Kundry up to 10.4 μl L^{-1} g^{-1} sprouts 3 days after the start of the experiment. TC130 sprouts build up a much lower ethylene concentration which levels out at 3 μl L^{-1} g^{-1} sprouts after 3 days.

Physiologically older sprouts of classical cultivars can produce up to 255 μl L^{-1} g^{-1} sprouts in 2 days (e.g. cultivar Ajax in figure 3). Whereas old TC130 sprouts still have a low ethylene production of 3 μl L^{-1} g^{-1} sprouts. The ethylene production determinations clearly show that the LSL inbred line TC130 and the derived Spr 110 have low, non-climacteric ethylene production, while classical varieties with a short shelf life have a climacteric ethylene production.
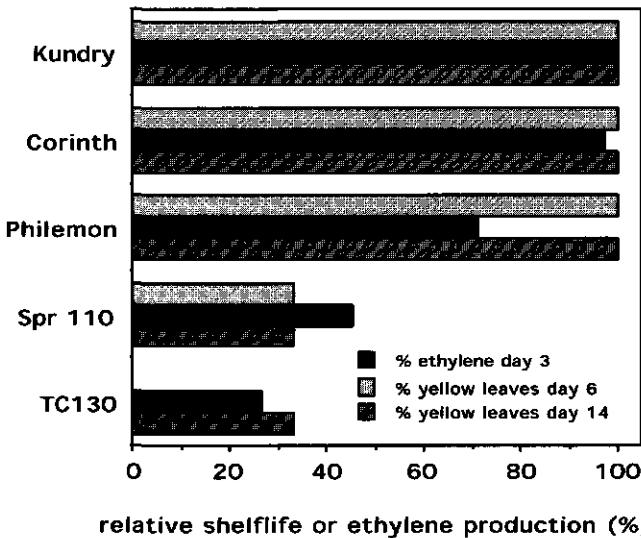

relative shelflife or ethylene production (%)

Figure 5. The relationship between the relative ethylene production of buttons of Brussels sprout cultivars and the relative number of yellow leaves 6 and 14 days after picking. The relative ethylene production is expressed as % of the highest production value (Kundry: 10.4 μl L^{-1} g^{-1}), the relative number of yellow leaves is expressed as a % of the highest number of yellow leaves observed (6 wrapper leaves).

In figure 5 the relationship is presented between the relative ethylene production of buttons from the cultivars and the relative number of yellow leaves observed. The high autocatalytical ethylene production by buttons of classical cultivars and their quick deterioration is probably a causal relationship. The ethylene insensitivity of the buttons of TC130 and Spr 110 coincides with a relatively low ethylene production and a lack of senescence.

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Shelf Life of Classical Sprouts can be Extended by Treatments with GA $_3$ and Zeatin.

The quick deterioration of sprouts from classical cultivars such as Corinth and Ajax is presumably related to their sensitivity to the hormone ethylene. The phytohormones cytokinin and gibberellin have the capacity to delay senescence in various (horticultural) crops (Kappers et al., 1998; Downs et al., 1997). Sprouts from TC130, Ajax and Corinth were picked and transferred with their feet (part by which the sprout is attached to the plant) onto water agar with varying concentrations of hormones to study the influence of these hormones on the senescence process of sprouts over 12 days. The time course of leaf yellowing on detached sprouts of the cultivars Corinth, Ajax and TC130 after the various treatments is presented in figures 6A-C. Sprouts from Corinth and Ajax quickly turned yellow on water agar, but at a slower rate to that observed after storage in ambient air at room temperature as presented in figure 2 and 3, probably because the sprouts can, unlike in the previous experiments, take up water from the water agar. Sprouts from TC130 stayed green on water agar. The percentage of yellow leaves on sprouts from Corinth and Ajax was considerably lower on day 9 after a treatment with GA$_3$ or zeatin. Corinth sprouts responded somewhat better to GA$_3$ than to zeatin, sprouts from Ajax had an equal response to both hormones. TC130 sprouts did not respond to treatment with any hormone and remained green as they did on water agar. The influence of zeatin and GA$_3$ faded away between 9 and 12 days.

Sprouts from Corinth and Ajax incubated on agar containing a combination of zeatin and GA$_3$ did not turn yellow during the whole period of 12 days and displayed a comparable level of shelf life to TC130 sprouts without any hormone treatment. The results of this experiment suggest that the expression of the LSL trait in sprouts from TC130 might be based on an alteration in the biosynthesis or accumulation of cytokinin and gibberellin or on an increased sensitivity of TC130 sprouts to these hormones. The results of these experiments do not exclude that cytokinin and gibberellin independently affect the shelf life of sprouts. The rate of yellowing of sprouts from the cultivars Corinth, Ajax and

TC130 was not affected by treatment with the auxin IAA. Sprouts from the three cultivars showed the same response as observed on water agar (data not shown).

Leaves of TC130 Plants are also Ethylene Insensitive

The expression of the LSL trait in TC130 is not restricted to sprout buttons only. Detached leaves from young Corinth and Ajax plants have a higher rate of yellowing compared to TC130 leaves when fed with 100 mg/L Ethrel via the petiole. Figure 7 shows the rate of yellowing of the third leaf of young plants from the cultivars during an incubation period of 7 days on a nutrient solution with 0 and 100 mg/L Ethrel. Ajax leaves started to turn yellow two days after the start of the experiment and were totally yellow after 5 days. Corinth leaves turned yellow at a lower rate but also were totally yellow after 7 days. TC130 leaves were only yellow for 15% of the leaf blade area at the top of the leaf one week after the start of the experiment.

So, leaves from TC130 had the capacity to delay the Ethrel stimulated yellowing process for an extra 5 days compared to leaves from classical cultivars. The TC130 leaves of figure 7 turned yellow in the second week of the experiment. The yellowing rate of leaves from older TC130 plants decreases, unlike the leaves from classical cultivars, further with plant age. The fifth leaf from 5 months old TC130 plants does not turn yellow when exposed to 100 mg/L Ethrel via the petiole (data not shown). TC130 leaves from these plants start to form roots on the cut surface of the petiole close to the vascular bundles about two weeks after exposure to Ethrel containing nutrient solutions. Root formation in the presence of Ethrel is exclusively observed on petioles of TC130 and derived cultivars and not on petioles from classical cultivars lacking extended shelf life.
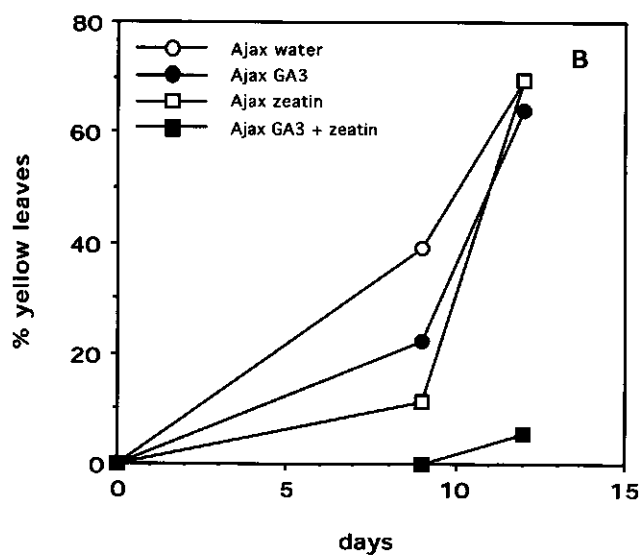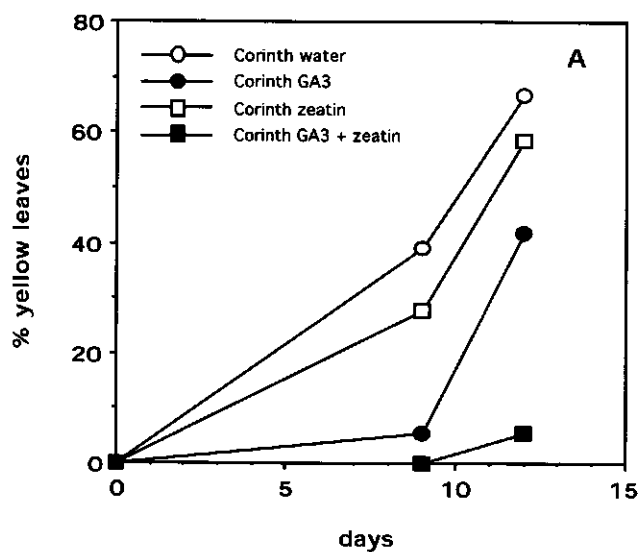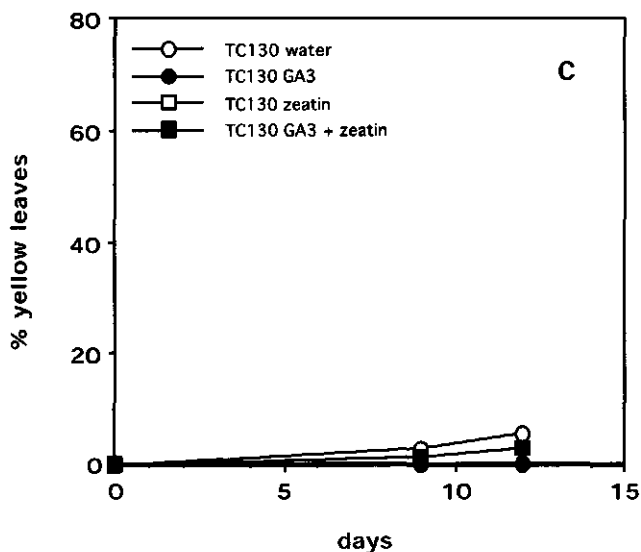
Figure 6. The percentage of yellow leaves on sprouts of the cultivars Corinth (panel A), Ajax (panel B) and TC130 (panel C) during an incubation of 12 days on water agar containing various hormones. Sprouts from the cultivars Ajax and Corinth and the LSL inbred line TC130 were harvested, disinfected with 1% (w/v) sodium hypochlorite and placed on 1% (w/v) water agar or water agar with 5 mg/L zeatin, 1 mM GA3 and a combination of 5 mg/L zeatin and 1 mM GA3 and kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room. The expression of LSL was scored as the number of yellow wrapper leaves on four sprouts 9 and 12 days after the start of the experiment and expressed as a percentage of the total number of wrapper leaves present on the sprouts.

TC130 Leaves are Resistant to the Auxin 2,4-D and the Auxin Transport Inhibitor HFCA.

Root formation of plants is under the control of auxin and probably mediated by the hormone ethylene (Ecker, 1995; Ruegger et al., 1997). The root forming capacity of TC130 petioles at Ethrel concentrations that promote senescence in leaves of classical cultivars might be related to an unique auxin mediated response. Detached leaves from TC130 and the broccoli cultivar Emperor, which have a comparable response to Ethrel as Corinth and Ajax leaves, were evaluated for the rate of yellowing as a
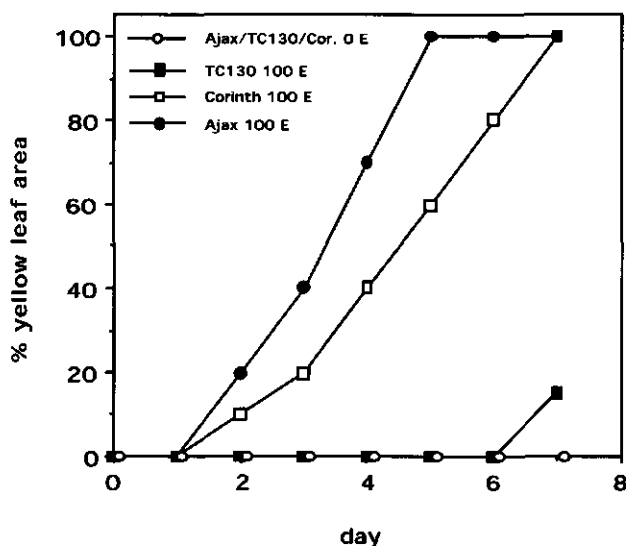
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Figure 7. The yellowing of detached third leaves from Corinth, Ajax and TC130 plants with an age of 5 weeks during an incubation period of 7 days on a nutrient solution with 0 and 100 mg/L Ethrel. The nutrient solutions were taken up via the petioles. The leaves were kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room during the experiment and evaluated for the rate of yellowing for 7 days after the start of the experiment. Yellowing was scored as a percentage of the total leaf blade area. Abbreviations: 0 E = no Ethrel, 100 E = 100 mg/L Ethrel, Cor. = cv Corinth.

function of the concentration of 2,4-D in the nutrient solution. The auxin 2,4-D was used for the induction of ethylene production, a classical auxin response of plants (e.g. Mito and Bennett, 1995; Woeste et al., 1999), and promotion of senescence. Figure 8 shows the % of yellow leaf area of TC130 and Emperor leaves as marker for senescence at variable 2,4-D concentrations in the nutrient solution after an exposure of 7 days. TC130 leaves did not respond to any 2,4-D concentration, continued leaf growth and expansion, and maintained their dark green colour. Emperor leaves however turned yellow and died in the presence of 2,4-D in a dose-dependent way. TC130 leaves apparently are insensitive to senescence promoting 2,4-D concentrations. The promotion of senescence in Emperor leaves by 2,4-D was not due to vascular

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callus mediated inhibition of nutrient uptake by the petiole as the uptake of nutrient solution by leaves was almost constant throughout the duration of the experiment. The induction of senescence by 2,4-D was also observed in Ajax and Corinth leaves (data not shown).
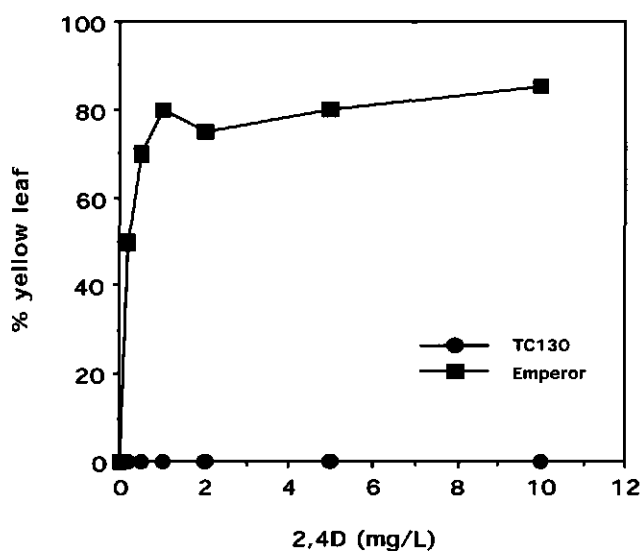

Figure 8. The yellowing of TC130 and Emperor leaves as a function of the 2,4-D concentration during an incubation period of 7 days on a nutrient solution containing 0 to 10 mg/L 2,4-D. The leaves were exposed to the nutrient solutions via the petioles. The leaves were kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room during the experiment and evaluated for the rate of yellowing 7 days after the start of the experiment. Yellowing was scored as a percentage of the total leaf blade area.

The stimulation of yellowing in detached Emperor leaves by 2,4-D containing nutrient solutions raised the question whether accumulation or trapping of the *de novo* biosynthesized auxin in leaves could also trigger senescence. Auxin accumulation in detached leaves can be enhanced by the inhibition of polar auxin transport with specific inhibitors (Rodrigues-Pousada et al., 1999). Figure 9 shows the response of detached Emperor and TC130 leaves to exposure of 10 μM of the polar auxin transport inhibitor 9-hydroxy-fluorone carboxylic acid (HFCA) in the

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nutrient solution for 2 weeks. Emperor leaves turned yellow in the presence of 10 μM HFCA. TC130 leaves were not affected by HFCA and remained green during the treatment. Inhibition of polar auxin transport apparently leads to auxin-stimulated yellowing in detached leaves of classical varieties. The yellowing of leaves was observed in particular at the top of the leaf blades, the leaf part with the highest auxin biosynthesis capacity.
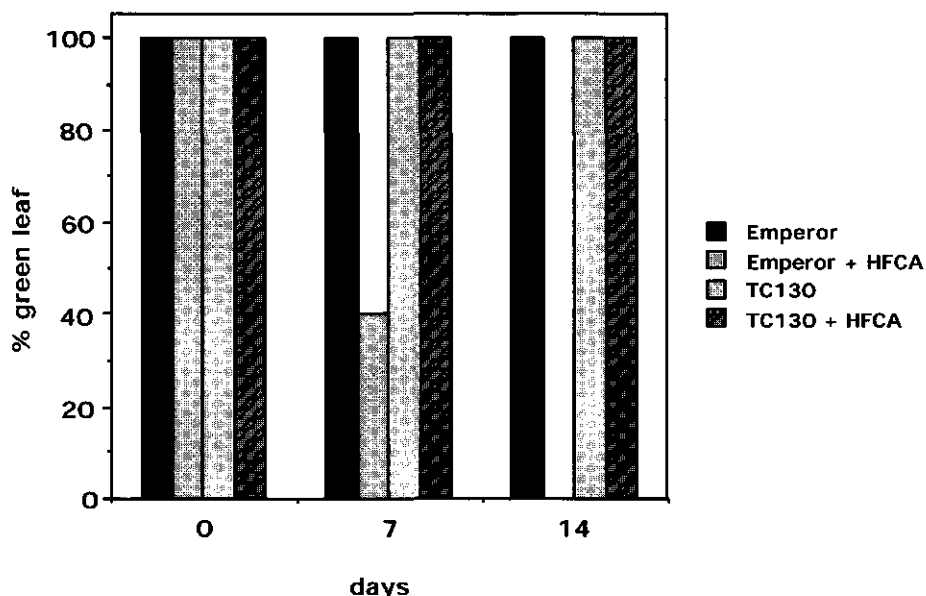
Figure 9. The yellowing of TC130 and Emperor leaves during a culture period of 14 days on a nutrient solution with 0 and 10 μM HFCA. The leaves were subjected to the nutrient solutions via the petioles. The leaves were exposed at 20 °C with a 16 hr photoperiod (10 klux) in a growth room during the experiment and evaluated for the rate of yellowing 7 days after the start of the experiment. Yellowing was scored as a percentage of the total leaf blade area.

Shelf Life of TC130 Sprouts is Related to a High Cytokinin Content.

The extension of shelf life of sprouts from the classical cultivars Corinth and Ajax by an incubation with zeatin and GA$_3$ suggests that the LSL of TC130 sprouts might be related to a high content of active cytokinins or GAs. The positive role cytokinins and GAs

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play in the maintenance of shelf life of sprouts was already proposed by Thomas (1977). The putative role cytokinins might play in the extension of shelf life was studied in 2 inbred lines and 4 Brussels sprout varieties with maximal variation in shelf life. Table 1 gives an overview of the cytokinin content and distribution in the inbred lines and cultivars and their shelf life after a treatment with 100 mg/L Ethrel. The cytokinin content of sprouts is predominantly comprised of zeatin-type cytokinins in the zeatin-riboside configuration.

Table 1. Cytokinin content and shelf life in sprouts of six Brussels sprout inbred lines and cultivars varying in shelf life. Cytokinins are categorized in zeatin-type, dihydrozeatin-type (DHZ) and iso-pentenyl-type (iP+iPA) cytokinins. The content and distribution of the various cytokinins was determined on lyophilized sprout samples according to Motyka et al. (1996) and expressed in pmol per g fresh matter. Shelf life of the sprouts is defined as the period in which sprouts from the inbred lines and cultivars stay green after a spray with 100 mg/L Ethrel.

cultivar	shelf life	zeatin		DHZ		iP + iPA	sum
		free base	riboside	free base	riboside		
	(days)	---pmol/gfw---		---pmol/gfw---		pmol/gfw	pmol/gfw
Spr 105	14	2	53	12	7	118	192
TC130	21	2	159	4	12	55	232
Adonis	7	1	67	4	13	22	107
Tavernos	8	1	78	2	10	13	104
DC69	11	1	137	2	7	5	152
Ajax	5	1	86	2	10	3	102

The sum of zeatin, dihydrozeatin and iso-pentenyl adenine derived cytokinins and corresponding ribosides in buttons of the cultivars and inbred lines is highly significantly correlated with their shelf life ($n = 6$, $r^2 = 0.96$, $p<0.01$). Figure 10 shows the linear relationship between the sum of cytokinins in sprouts of the 6 cultivars and inbred lines and the shelf life.
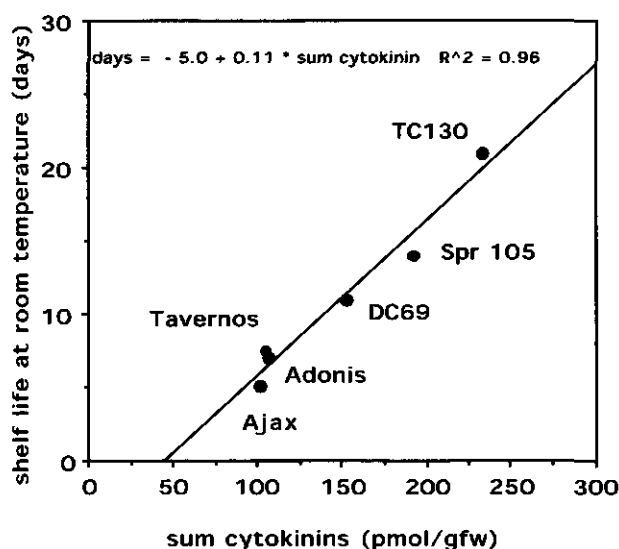
Figure 10. The relationship between the sum of zeatin, DHZ and iP type cytokinins and the shelf life of 6 sprout cultivars. The determination of cytokinins and the screening of shelf life have been conducted according to the legend of table 1.

TC130 seedlings develop normally in the presence of an overdose cytokinin.

A high cytokinin content in plant(parts) in general triggers their autocatalytic conjugation and oxidation (Kaminek at al., 1997; Auer et al., 1999) with transient cytokinin profiles in plant tissues as a result. Plants with a high cytokinin production have according to the former reasons a higher cytokinin inactivation capacity than low cytokinin producers. Seedlings of the LSL line TC130 and the derived F1-hybrid Spr 105, with a high cytokinin content (see table 1), display almost normal root development, hypocotyl elongation and cotyl expansion in the presence of an overdose of 15 mg/L kinetin. Seedlings from the cultivar Ajax, with a low cytokinin content, are sensitive to this kinetin concentration and form short hairy roots and short hypocotyls under these circumstances. The apical hook of Ajax seedlings became necrotic about 2 weeks after sowing. The observations of the kinetin experiments are presented in table 2. Brussels sprout

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seedlings from LSL lines with a high cytokinin content have, unlike seedlings from low cytokinin lines, the capacity to resist a high dose of cytokinin probably because of their high cytokinin inactivation capacity.

Table 2. Development of Brussels sprout seedlings in the presence of a high kinetin concentration. Seedlings were germinated on water agar, containing 0 or 15 mg/L kinetin, in a growth room at 20 °C with a 16 hr photoperiod (10 klux) for a period of 2 weeks and evaluated for developmental parameters such as germination, root development, hypocotyl elongation and cotyl expansion. Abbreviation: a.h. = apical hook.

cultivar	kinetin mg/L	germination	root development	hypocotyl elongation	cotyl expansion	remarks
Ajax	0	normal	normal	normal	normal	-
	15	normal	inhibited	inhibited	inhibited	necrosis a.h.
TC130	0	normal	normal	normal	normal	-
	15	normal	almost normal	almost normal	normal	-
Spr 105	0	normal	normal	normal	normal	-
	15	normal	almost normal	almost normal	normal	-

The Germination of Seeds from TC130 and Derived F1-Hybrids is Insensitive to Papaverin.
The insensitivity of TC130 sprouts and leaves to ethylene, 2,4-D, kinetin and the polar auxin transport inhibitor HFCA on one hand and the high level of cytokinins in sprouts on the other hand suggest that the expression of LSL of the inbred line is somehow related to an altered hormone balance in favour of the trait. The cytokinin-auxin ratio (CAR) of sprouts might affect the expression of shelf life (see introduction) and can, along with cytokinin and auxin import and export, be controlled by conjugation and oxidation of cytokinins and auxins (Kaminek at al., 1997, Eklof et al., 1997). Papaverin, an inhibitor of $N^{7,9}$-glucosylation of cytokinin-ribosides (Tao et al., 1991) was
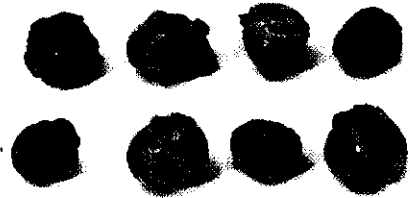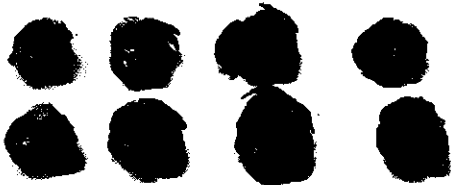
4A: TC 130

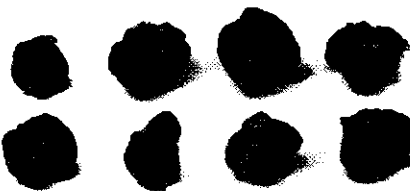4B: TT 29

4C: TC 59

4D: TC 25

4E: DC 69

4F: TC 55

+E

−E

4G: TC 130   TT 29   TC 59   TC 55
1 week post-harvest

+E

−E

4H: TC 130   TT 29   TC 25   DC 69
1 week post-harvest

+E

−E

4I: TC 130   TT 29   TC 25
2 weeks post-harvest

applied to study the possible role of cytokinin conjugation on the expression of LSL. Germinating seeds have a high uptake capacity for water and dissolved compounds during the imbibition phase and are therefore ideal to study the influence of compounds such as papaverin which interfere with the hormone balance during early seedling development. Seeds from inbred line TC130 and derived F1-hybrids show normal radicle protrusion, hypocotyl elongation and cotyledon expansion when germinated on water agar containing 5 mM papaverin whereas seeds of classical sprout inbred lines and cultivars fail to germinate under such conditions. Embryos from seeds of TC130 and related F1-hybrids are insensitive to papaverin which is indicative of a high cytokinin conjugation activity. Cytokinin conjugation seems to be low in germinating seeds from classical inbred lines and F1-hybrids as inhibition of conjugation by papaverin is lethal. The high cytokinin conjugation activity in seeds of TC130 and related F1-hybrids seems to be necessary to prevent the accumulation of a toxic overdose of active cytokinins. The cytokinin conjugation capacity of tissues is apparently related to the content of active cytokinins.

TC130 and Derived F1-Hybrids Display Low Polar Auxin Transport Capacity.
The polar auxin transport system of plants is reported to play an important role in the regulation of an array of developmental processes including apical dominance, leaf abscission and lateral root formation (Ruegger et al., 1997).

Legend to figure 4. The senescence process in sprouts of LSL and classical sprouts after water and Ethrel sprays. Sprouts from lines TC130, TT29, TC59, TC25, DC69 and TC55 were picked freshly (figures 4A to 4F respectively), sprayed with water and 100 mg/L Ethrel, and subsequently evaluated for a period of 2 weeks. Figure 4G presents from left to right sprouts from respectively TC130, TT29, TC59 and TC55 sprouts 1 week after a water (bottom containers: -E) or Ethrel spray (top containers: +E). Figure 4H presents from left to right TC130, TT29, TC25 and DC69 after the same treatments displayed with the same design. Figure 4I is showing TC130, TT29 and TC25 sprouts 2 weeks after the former treatments.

The dormancy of axillary buds, such as Brussels sprouts, is maintained by polar auxin transport. The uptake of assimilates and hormones like cytokinins from respectively the leaves and roots is probably controlled by polar auxin transport activity (Bangerth, 1989). A putative relationship between the expression of shelf life in Brussels sprouts and the polar auxin transport capacity of Brussels sprouts inbred lines and F1-hybrids was studied in germination tests on water agar and the polar auxin transport inhibitor HFCA. Seedlings from classical and LSL sprouts show typical but different responses to HFCA. Cotyls and hypocotyls of classical cultivars such as Ajax and Corinth develop normally at a concentration of up to 20 μM HFCA but form less lateral roots at a HFCA concentration of 5 μM or higher. Seedlings of TC130 and related F1-hybrids, however, loose dose-dependently their gravitropical response in the presence of HFCA and show dramatic inhibition of primary and lateral root formation. The loss of the gravitropical response of seedlings can be observed as a decrease of the angle between the hypocotyl and the horizontal from 90° in the absence of HFCA to approximately 20° in the presence of 10 μM HFCA and 0° in the presence of 20 μM HFCA. In figure 11, the response of LSL seedlings (upper panel) and classical seedlings (lower panel) to 10 μM HFCA is expressed diagramatically. A low capacity of polar auxin transport is linked with the expression of LSL in TC130 and derived F1-hybrids.

DISCUSSION.

Expression of LSL and Ethylene Responses in Seedlings and Edible Parts of Inbred Line TC130

In this paper we have demonstrated that the expression of the LSL trait in sprouts from the Brussels sprout inbred line TC130 is correlated with multiple hormone responses. Classically, the senescence of plant parts such as leaves and flowers can be triggered by a treatment with ethylene (Ecker, 1995). Harvested sprouts from inbred line TC130 are insensitive to Ethrel concentrations in the mg/L range, concentrations which cause the quick deterioration of almost all plant parts of other lines treated this way.
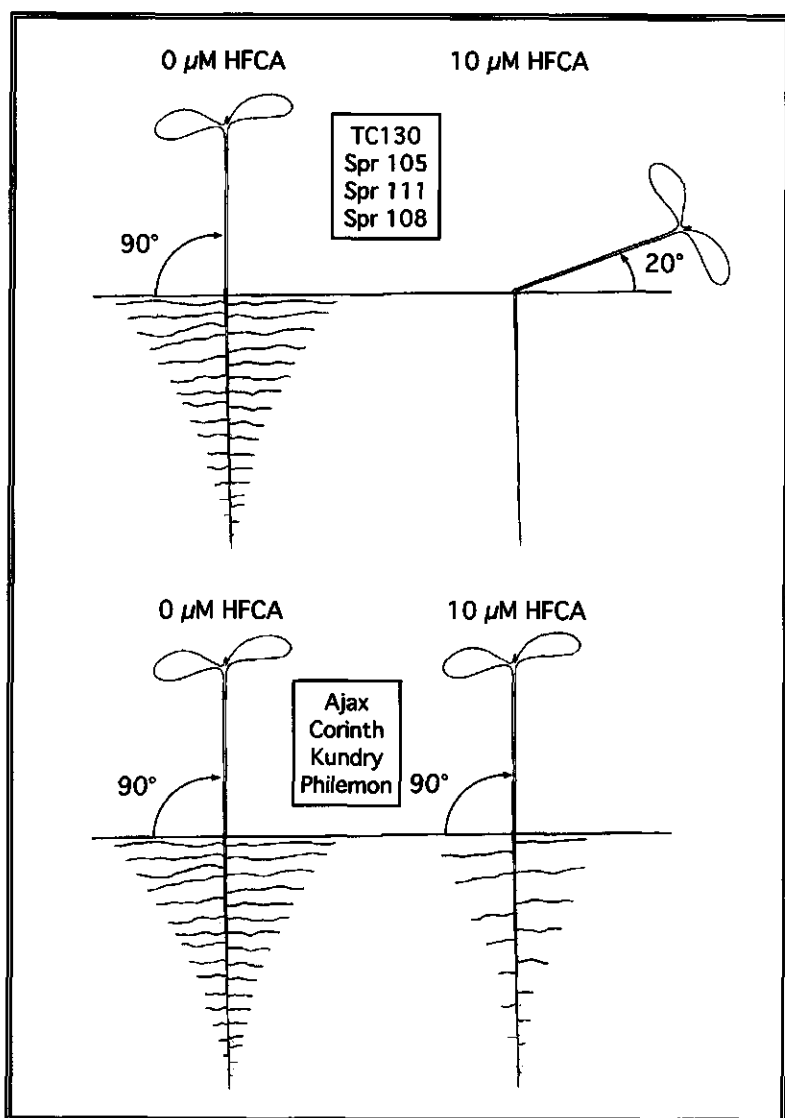
Figure 11. Schematic overview of the response of LSL seedlings (upper panel) and classical seedlings (lower panel) during germination on water agar containing 0 and 10 μM HFCA. The gravitropic response of seedlings was used as a marker for polar auxin transport in the hypocotyl and measured as the average angle (in $^{\circ}$) between seedling hypocotyls and the horizontal. Seedlings with an angle of 90° with respect to the horizontal on 10 μM HFCA have a high capacity of polar auxin transport, seedlings with a small angle (e.g. 20°) have a low capacity.

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Surprisingly seedlings from inbred line TC130 display a more pronounced triple response than a non-LSL line in the presence of ethylene (figure 1), an indication that the ethylene receptor and signal transduction pathway are functioning normally. The discrepancy between the presence of the triple response in TC130 seedlings and the expression of shelf life in sprouts and leaves of TC130 plants confirms the finding of Klee and Romano (1994) that only competent plant parts will senesce in response to ethylene.

Not only the sprouts but also the leaves from inbred line TC130 are resistant to a high dose of Ethrel (figure 7). The ethylene insensitivity trait is apparently expressed in leaves which are positioned on both the stem and lateral shoots (sprouts are per definition dormant lateral shoots). The expression of LSL in the leaves was used to study the senescence process of Brussels sprout lines and cultivars before sprouts were formed at an early stage of plant development.

Non-Climacteric Ethylene Production in TC130 Sprouts

Sprouts from inbred line TC130 have a reduced ethylene production, which is less than 30% of the production of classical sprouts. The low ethylene production of TC130 sprouts is in contrast to the high ethylene production of the ethylene insensitive etr mutant in *Arabidopsis thaliana* which lacks a negative feedback control of ethylene biosynthesis (Bleecker et al., 1988). The regulation of ethylene production of plants can be categorized in system I and system II type of regulation (Lelievre et al., 1997). System I operates in both climacteric and non-climacteric tissues and can be recognized by the down-regulation of ethylene production by external ethylene. The system II type of regulation is found in climacteric plant parts whose ethylene production is stimulated by external ethylene. TC130 sprouts have a low and non-climacteric system I type of ethylene production and fail to initiate the autocatalytical system II type of ethylene biosynthesis (figure 5).

According to Ecker (1995) the rate limiting step of ethylene biosynthesis is the conversion of SAM (S-adenosyl-L-methionine) to ACC (1-amino-cyclopropane-1-carboxylic acid) by the enzyme ACC synthase (ACS). The ACC-synthase gene family consists of at least 7 members ACS1-ACS7 in *Arabidopsis thaliana* (Liang et al.,

1992 and 1995; Arteca and Arteca, 1999; Woeste et al., 1999) and 9 members in tomato (Lelievre et al., 1997) of which some are expressed in fruits. Some ACS genes, such as tomato ACS2 and ACS4 are positively regulated by ethylene (Lincoln et al., 1993) and part of the system II ethylene biosynthesis route. Others, such as *Arabidopsis thaliana* ACS4 and ACS5, are under the control of auxin and cytokinin respectively (Abel et al., 1995; Vogel et al., 1998a and b). ACS5 seem to belong to the system I ethylene biosynthesis route with negative feedback control over its ethylene production. ACS catalyzed ethylene production is controlled by developmental and environmental factors (Klee and Romano, 1993; Lanahan et al. 1994). The content of auxin and cytokinin or cytokinin to auxin ratio (CAR) in plant tissues might be the developmentally regulated parameters responsible for regulation of system I or system II mediated ethylene production. A high CAR is associated with system I type of ethylene production, a low CAR with the system II type. The gradient-wise distribution of the auxin IAA and various cytokinins in a model plant such as tobacco (Eklof et al., 1997) supports the possible role these hormones might play in the control of ethylene production. The role of auxin and cytokinin in relation to ethylene production and LSL will be addressed later in this section.

Our findings suggest that the ethylene insensitivity exhibited by TC130 is more effective with respect to the prevention of ethylene triggered senescence than the ethylene receptor mediated ethylene insensitivity observed in ethylene receptor mutants. Tomato or *Arabidopsis thaliana* plants expressing respectively the nr (Lanahan et al., 1994) or etr1 (Oh et al., 1997) mutation suppress several ethylene related physiological responses but cannot delay senescence in detached plant parts as effectively as TC130 plants. TC130 leaves and sprouts presumably have a longer shelf life because of the suppression of both ethylene production and perception while nr and etr1 plants are only insensitive for responses mediated by one defective receptor but still keep the capacity to sense ethylene via other receptors (Hua et al., 1995; Lashbrook et al., 1998).

Influence of Cytokinin and GA on the Expression of LSL
It is interesting to note that classical sprouts without a long

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shelf life express LSL after an incubation with the cytokinin zeatin, GA_3 or a combination of these hormones (figure 6). The results of the hormone incubation experiment indicate that the expression of shelf life is affected by cytokinins and gibberellins. Gan and Amasino (1995) have clearly demonstrated that the senescence machinery of plant parts can be inhibited by the synthesis of small amounts of cytokinin in their SAG12-IPT system in tobacco. Apparently depletion of cytokinins is a developmental signal for the initiation of the senescence process.

The expression of shelf life in sprouts of various cultivars is highly correlated to the content of cytokinins (figure 10). Several lines of evidence confirm the role of cytokinins in the expression of shelf life of plant parts and the standing ability or life span of plants. The det2 mutant of Arabidopsis thaliana displays prolonged shelf life in detached leaves due to an altered response to cytokinin (Chory et al., 1994). The increased life span of the amp mutant is correlated with an elevated content of cytokinins (Chaudhury et al., 1993; Chin-Atkins et al., 1996). The degradation of chlorophyll of Brussels sprouts is inversely and co-linearly related with their cytokinin content (Thomas, 1977). TC130 sprouts contain more than twice the content of cytokinins than sprouts from classical cultivars such as Ajax, Tavernos and Adonis (figure 10). Except for Spr 105, the main cytokinin observed in sprouts is zeatin-riboside (table 1), the transport cytokinin which is synthesized in the roots and transported to target organs via the xylem by means of transpiration and root pressure (Bangerth, 1994; Farkhutdinov et al., 1997; Dieleman et al., 1997; Beveridge et al., 1997).

The accumulation of a high content of active cytokinins in the sprouts can be controlled by various mechanisms but is primarily dependent on a high uptake rate and a low rate of inactivation. The uptake of cytokinins from the xylem is not understood well but probably mediated by the membrane bound enzyme adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) (Burch and Stuchbury, 1987; Kaminek et al., 1997). Cytokinins are, depending on their structure, inactivated by means of oxidation and conjugation. Zeatin and iP type cytokinins, in contrast to dihydrozeatin and aromatic type cytokinins, can be oxidized by the

enzyme cytokinin oxidase, which is transiently expressed in response to increased cytokinin levels (Kaminek et al., 1997; Auer et al., 1999). Cytokinins can be N-conjugated by means of 7- and 9-glucosylation and alanine conjugate formation (Letham and Palni, 1983) or O-glycosylated to yield O-glucosyl or O-xylosyl cytokinins (Auer, 1997; Martin et al., 1997). The physiological relevance of cytokinin conjugation is clearly demonstrated by the group of Letham and co-workers in a series of papers in which cytokinin conjugation is inhibited by a selection of specific inhibitors (e.g. Tao et al, 1991). Our findings that TC130 seedlings can resist a high dose of kinetin and the cytokinin conjugation inhibitor papaverin, indicators for a high cytokinin conjugation rate, suggest that the accumulation of cytokinin in TC130 might be due to a high uptake rate. Our experiments with papaverin, a specific inhibitor of cytokinin 9-glucosylation, also show that the inactivation of cytokinins by 9-glucosylation is needed for proper growth and differentiation of seedlings during early stages of development.

The expression of LSL in inbred line TC130 might be related to particular components of the cytokinin signal transduction pathway, in analogy to the constitutive ethylene response observed in ctr seedlings in *Arabidopsis thaliana* (Kieber et al., 1993). At present very little is known with respect to cytokinin perception and signal transduction pathways and their interaction with ethylene production and signalling. Recently a putative cytokinin receptor (Kakimoto, 1996) and two cytokinin response regulators with a transient expression pattern (Brandstetter and Kieber, 1998) have been identified in *Arabidopsis thaliana* which seem to play a role in the cytokinin signal transduction pathway. The regulators are activated by 1 to 50 μM BAP, a relatively high concentration range when compared to the cytokinin concentration of 0.23 μM (230 pmol gfw^{-1}) cytokinins in TC130 sprouts (Table 1). The role of these regulators in the expression of shelf life in sprouts can now be studied as the sequences of the homologous genes are available.

The senescence delaying action of GAs has extensively been studied in leaves of Alstroemeria (Kappers et al., 1997 and 1998). GA_1 and GA_4, in synergy with phytochrome, delay senescence in

Alstroemeria leaves. The underlying mechanism of GA mediated inhibition of senescence remains to be solved. GAs seem to play a cosmetic role by preventing the degradation of chlorophyll but cannot inhibit other senescence related processes.

Stimulation of Senescence in Sprouts by Auxin

The observation of the senescence-delaying capacity of zeatin in sprouts raised the question whether auxin could stimulate senescence. Auxin is involved in all aspects of plant growth and development and controls many physiological processes in plants including ethylene production (reviewed by Hobbie, 1998). The key auxin in plants is indole-3-acetic acid (IAA) which mainly occurs as ester-linked or amide-linked conjugates. Mutations in the SUPERROOT/ROOTY/HOOKLESS3 gene of *Arabidopsis thaliana* lead to accumulation or overproduction of IAA and derived conjugates (Boerjan et al., 1995; King et al., 1995; Lehman et al., 1996, Celenza et al., 1995). Pleiotropic aberrations observed in these mutants resemble ethylene triggered physiological responses of plants exposed to low ethylene concentrations. In contrast, the auxin resistant mutants such as axr1 (Estelle, 1996), aux1 (Pickett et al., 1990) and ein2 (Fujita and Syono, 1996) are all cross-resistant to ethylene. These results suggest an important role for auxin in the control of ethylene biosynthesis or signalling.

Auxin upregulates specific ACC synthase and ACC oxidase genes and ethylene production (Abel et al., 1995; Sitbon and Perrot-Rechenmann, 1997; Mito and Bennett, 1995; Peck et al., 1998). In our experiments the auxin 2,4-D stimulated senescence in leaves of classical Brussels sprout and broccoli cultivars but not in TC130 leaves (figure 8). The result of the 2,4-D incubation experiment suggests that TC130 leaves might lack auxin inducible ACS and ACO genes or are incompetent to senesce in response to auxin. The stimulation of expansion growth of leaf blades, another general auxin-dependent response, in the presence of 10 mg/L 2,4-D suggests that 2,4-D is taken up and distributed effectively in the leaf blade. The role of auxin in the induction of senescence in leaves was further substantiated by the observation that leaves from classical sprout and broccoli cultivars turn yellow after a treatment with a polar auxin transport inhibitor HFCA

(figure 9). Accumulation of *de-novo* synthesized auxins in the leaf blade by inhibition of polar auxin transport lead to the induction of senescence in Emperor. However, TC130 leaves did not senesce after a treatment with HFCA and maintained growth and a dark green colour.

The experiments with auxin and HFCA and detached leaves from classical cultivars and the inbred line TC130 demonstrate that the auxin concentration or the ratio between auxin and other senescence affecting plant growth regulators play an important role in the induction of senescence. The expression of LSL in the inbred line TC130 might be controlled by a disruption in an auxin mediated response. Molecular characterization of the auxin inducible ACS and ACO genes, the AXR1 gene and the genes for polar auxin transporters such as PIN1 and TIR3 in the inbred line TC130 will elucidate whether mutations in one of these genes are linked to the expression of the LSL trait. The sensitivity of TC130 roots and hypocotyls to 2,4-D and ethylene during germination tests suggests, however, that the expression of LSL in the inbred line TC130 is not primarily controlled by a mutation in one of these auxin signalling genes.

The Impact of Interactions between Auxin and Cytokinin on the Expression of LSL

Sprouts from the inbred line TC130 have a significantly higher content of cytokinins. The content of cytokinin of plant tissues is controlled by the action of auxin in direct and indirect ways (Bangerth et al., 1994; Zhang et al., 1995; Eklof et al., 1997). Auxin is reported to stimulate the inactivation of cytokinins by means of oxidation in tobacco transgenic ipt expressing tissues (Zhang et al., 1995) and 7-glucosylation in *Dianthus zeyheri* (Crouch and Van Staden, 1995). The induction of senescence in detached leaves from classical Brussels sprout cultivars by 2,4-D and HFCA is probably triggered by auxin stimulated oxidation or conjugation of cytokinins (Kaminek et al., 1997) and the subsequent stimulation of ethylene production. Auxin mediated apical dominance also has a profound effect on the cytokinin content of plants. Li et al. (1995) demonstrated that removal of the apex, the main site for auxin biosynthesis, in pea plants resulted in a quick rise of the cytokinin content in the internode

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below the decapitation point, a process which could be reversed by the application of the auxin naphthyl acetic acid (NAA) on the cut end. The former findings suggest a role for the regulation of the cytokinin content of plant tissues by basipetal polar auxin transport. The interaction between polar auxin transport and cytokinin activity is further substantiated by Strabala et al. (1996), who demonstrated that cytokinin mediated responses in leaves of tobacco were enhanced by the polar auxin transport inhibitor NPA.

A Role for Polar Auxin Transport in the Expression of LSL

The expression of LSL in TC130 sprouts and derived cultivars is correlated with a low auxin transport capacity (figure 11). Hypocotyls of seedlings from our LSL inbred lines and cultivars loose their gravitropic response in the presence of the polar auxin transport inhibitor HFCA during germination and early seedling development. In plants, polar auxin transport is involved in an array of physiological processes such as gravitropism in shoots and roots (Fukaki et al., 1996; Estelle, 1996), maintenance of apical dominance (Bangerth, 1994), cell elongation (Gray et al., 1998), morphogenesis of plant organs such flowers and leaves (Okada et al., 1991), and the expression of LSL in our experiments. The expression of LSL in sprouts and leaves from the inbred line TC130 and derived cultivars seems to be due to the release of apical dominance as a consequence of a low polar auxin transport rate. The sink strength and related influx of assimilates and plant growth regulators such as cytokinins in lateral plant parts like sprouts and leaves is inversely related to the expression of apical dominance and controlled by the auxin transport rate from the apex. Recently Hamamoto et al. (1998) have demonstrated that the accumulation of auxin in tomatoes by means of inhibition of polar auxin transport in the peduncle of tomato clusters is resulting in a decreased sink strength in fruits, and leading to calcium deficient tomato fruits expressing auxin stimulated disorders such as puffiness. In contrast, increased auxin transport in developing wheat grains via the application of auxin containing gels on flag internodal lacunae enhances the grain photoassimilate and dry matter import (Darussalam and Patrick, 1998). In our experiments, Brussels sprout cultivars with a high

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auxin transport rate contained a low level of cytokinins, produced large quantities of ethylene and did not express a long shelf life. LSL varieties combined a low rate of auxin transport with a high cytokinin content and low ethylene production. We believe that the observed high level of cytokinins in LSL sprouts can be maintained because of a low polar auxin transport rate. A low polar auxin transport rate might favor the cytokinin content of lateral plant parts in several ways. An increased export of auxin from lateral tissues will increase their CAR and decrease the auxin stimulated oxidation and conjugation of cytokinins. A higher auxin export from lateral tissues will increase the inport of cytokinins and with that lead to a higher CAR.

Several other reports as well as our experiments describe interactions between the phytohormones auxin and ethylene and basipetal polar auxin transport. In *Arabidopsis thaliana* the pir1 and pir2 mutants, with inhibited polar auxin transport, have normal root elongation in the presence of the ethylene precursor ACC (Fujita and Syono, 1996) while this process is inhibited in the wild type. The polar auxin transport inhibitor NPA inhibits hypocotyl elongation in light-grown but not in dark-grown *Arabidopsis thaliana* seedlings (Jensen et al., 1998). Similar observations have been recorded for the influence of ethylene on hypocotyl elongation of seedlings. Ethylene triggers the triple response in dark-grown seedlings but stimulates hypocotyl elongation in the light (Smalle et al., 1997).

A Model for Hormone Mediated Expression of LSL

The hormonal characterization of the TC130 LSL inbred line has resulted in new insights into the control of shelf life in edible plant parts and identified a key role for auxin and cytokinin in the expression of this trait. The results from our experiments and recent achievements in the field of hormone research by other groups, as previously discussed in this section, indicate the existence of antagonistic routes with respect to ethylene mediated physiological responses during growth and development of plants. Ethylene driven processes are either supporting growth and differentiation or promoting senescence, ripening or deterioration of plant organs. Figure 12 presents a model for the interactions between the hormones auxin, cytokinin and ethylene
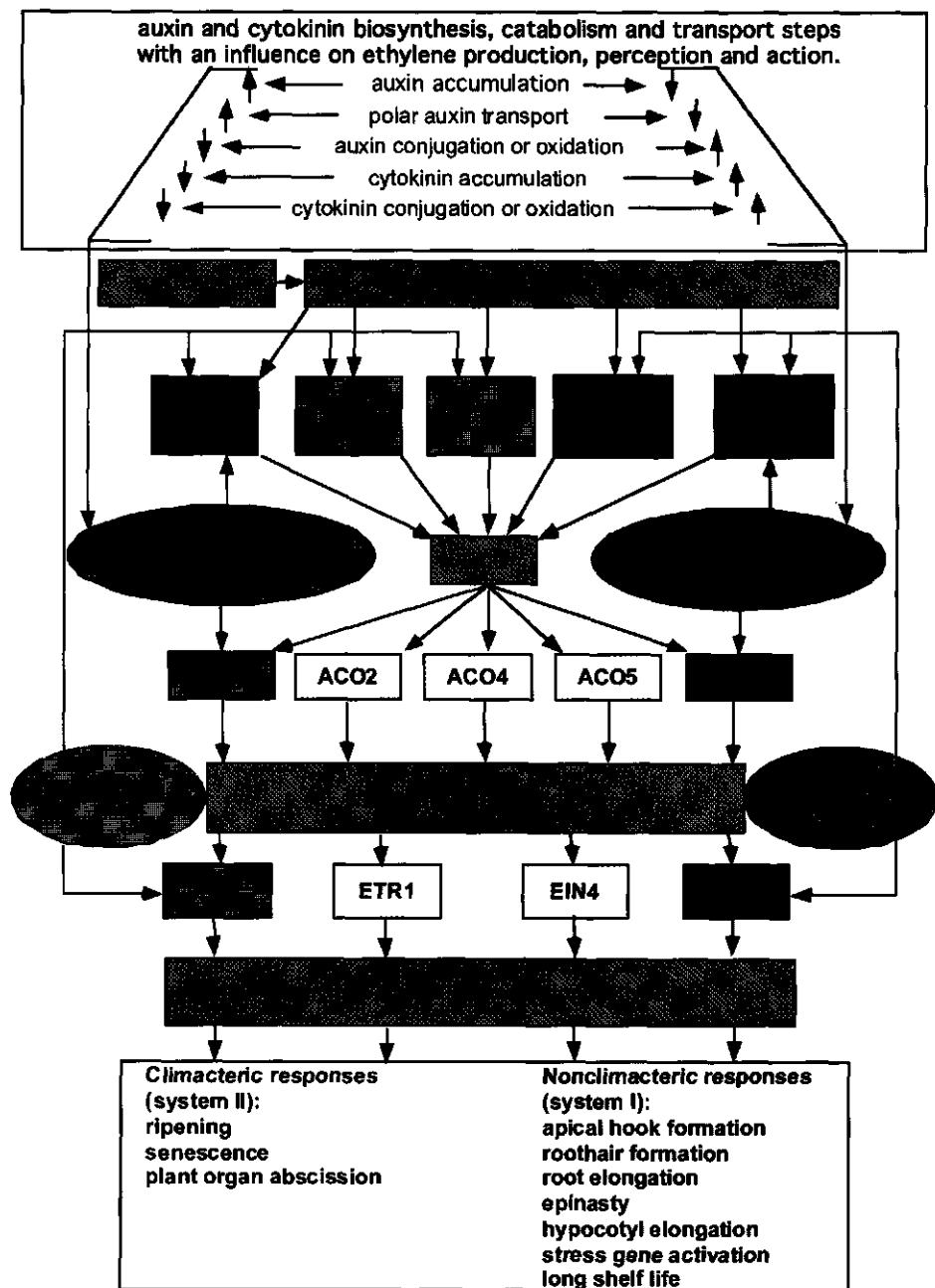
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Figure 12. CAR controlled ethylene production and ethylene dependent responses in plants (see legend on page 207 for more details)

in ethylene driven processes during plant growth and development based on the phenotype of inbred line TC130 and the results of recent papers. In our correlative model are ethylene production, perception and signal transduction under the strict control of the cytokinin/auxin ratio (CAR). Plant parts with a high CAR have controlled non-climacteric ethylene production via the green route (e.g. ACS5, ACO3, ETR2) which is mediating growth and differentiation related physiological responses. Plant parts with a low CAR have autocatalytical climacteric ethylene production via the red route (e.g. ACS4, ACS2, ACO1, ERS/NR) which is responsible for physiological processes such as ripening, senescence and deterioration of plant parts. Auxin and cytokinin play a distinct but different role in the control of ethylene production. The kinetics of the accumulation and inactivation of auxin and cytokinin are different. Auxin is efficiently inactivated by means of conjugation and oxidation at relative low concentrations (e.g. Eklof et al., 1997), cytokinins are transiently inactivated after their accumulation at relative high levels (Kaminek et al. 1997). Consequently, cytokinin accumulation and cytokinin inactivation will occur simultaneously and be related to ethylene production via the green route.

Figure 12. Model for the cytokinin/auxin ratio (CAR) modulated ethylene production and signalling pathways. The top panel of the figure depicts auxin and cytokinin biosynthesis, catabolism and transport steps with an influence on ethylene production and signalling. The blue boxes comprise general components of the ethylene biosynthesis and signalling pathways which seem not to be affected in TC130 plants. The grey boxes in the center of the figure comprise ACO and ACS genes/enzymes which are not well defined yet for hormone mediated regulation. The middle panel depicts on the left, in red, the ACO, ACS and ethylene receptor genes whose expression or enzyme activity is enhanced by a low CAR and/or ethylene. On the right, in green, the ACO, ACS and ethylene receptor genes are presented whose expression or enzyme activity is downregulated by a high CAR and/or ethylene. The panel at the bottom of the figure presents which non-climacteric (in green) and climacteric (in red) responses are triggered by CAR-modulated ethylene production and signalling

Auxin only will accumulate at low inactivation rates and consequently auxin accumulation has an opposite effect on ethylene production than auxin oxidation and conjugation.The maintenance of a high CAR seems to be the key condition for the expression of LSL in vegetative parts. Mutants with a high CAR like amp1 (Chaudhury et al., 1996) and our TC130 inbred line display standing ability at the plant level and long shelf life in detached plant parts. The role of auxin and cytokinin in the expression of LSL is just in an exploratory stage. The cloning and characterization of a cytokinin-sensitive ethylene-downregulated ACS gene (Vogel et al., 1998b) and an auxin-sensitive ethylene-upregulated ACS gene (Abel et al., 1995) in *Arabidopsis thaliana* and the LSL trait in the inbred line TC130 however indicate the high potential of CAR regulated ethylene production for the control of LSL in vegetable crops.

The involvement of cytokinin, auxin and ethylene in the regulation of shelf life and the mutual interactions between these phytohormones might explain why breeding for long shelf life in vegetable crops has been relatively unsuccessful in many crops. Long shelf life can only be obtained when all essential criteria for optimal expression are met. The complexity of shelf life regulation in terms of hormonal control is presumably the main reason why transgenic expression of a collection of ethylene production and response genes has not resulted in stable inheritable LSL genitors with an acceptable flavour and texture for consumers in tomato, melon and other climacteric crops. The characterization of the Brussels sprout LSL line is another step in the direction of the understanding of the complex control of shelf life expression.

METHODS

Plant Material.
The Brussels sprout inbred lines and F1-hybrids used in this study all originated from the breeding program of Novartis Seeds VFE BV in Enkhuizen, The Netherlands. The inbred lines and F1-hybrids were sown annually in January in multicell trays, young plants raised until April and planted in the field at the beginning of May.

Brussels sprouts were harvested from the end of August upto March in the following year depending on the earliness of the variety. Sprouts were picked for the experiments at maturity, the normal stage for consumption. Leaves were collected either from young plants at an age of 5 weeks or adult plants of 5 months for shelf life experiments with detached leaves. Brussels sprout plants of the various inbred lines and cultivars were grown according to standard commercial practice in the trial fields of Novartis Seeds BV at De Schermer, The Netherlands.

Selection of LSL

In the sprout breeding program parental inbred lines and F1-hybrids were evaluated for standing ability in the field as a first criterion for LSL. Sprouts from plants with possible expression of LSL could maintain a fresh and green appearance in the field far beyond the point of optimal maturity. Sprouts from such candidate LSL plants were picked halfway from the stem and used for the screening of LSL. Five sprouts per plant were sprayed with 100 mg/L Ethrel A (Ethephon) and placed in transparent plastic bags of 18*12 cm (length * width) and kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room. LSL was scored as the development of yellow wrapper leaves in the 2 weeks following spraying. The expression of LSL was, unless stated otherwise, evaluated 2 weeks after the Ethrel treatment and scored as the average number of yellow wrapper leaves on the sprouts on a scale between 0 (extreme LSL) and 6 (no LSL).

Evaluation of the Triple Response in Brussels Sprout Inbred Lines.

Seeds (20 seeds per inbred line per treatment) from the inbred lines TC130 (LSL) and TC25 (non-LSL) were sown in 20*10*5 cm (length*width*height) white polycarbonate boxes with a transparent lid on 3 layers of paper which were saturated with water diluted Ethrel solutions in a concentration range up to 15 mg/L. The seeds were allowed to germinate in darkness for 7 days at 20 °C and subsequently evaluated for the classical three ethylene-mediated responses: inhibition of root formation, inhibition of hypocotyl elongation and exaggeration of the apical hook. The hypocotyl length was used as the most convenient marker for the triple response and determined as a function of the

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Ethrel concentration the seeds were germinated on. The hypocotyl length was expressed as the average of ten uniform seedlings in an absolute (in mm) and relative (as % of the hypocotyl length on water) way.

Determination of the Optimal Ethrel Dose for the Selection of LSL

Sprouts from the inbred line TC130 and the cultivar Corinth were picked at the edible stage and sprayed with 0, 50, 100 and 200 mg/L Ethrel to study the optimal Ethrel dose for the selection of LSL. After spraying the sprouts were placed in transparent plastic bags of 18*12 cm (length * width) and kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room. The expression of LSL as function of the various Ethrel concentrations was scored as described previously.

Ethylene Production of Brussels sprouts.

The ethylene production of sprouts was determined in the headspace of closed jars over the course of 3 days using a gas chromatograph (Shimadzu GC8A, Japan) equipped with a Carbopack column (BHT 60-80M, O.D. 1/8", 6 feet length, Supelco, USA). Glass jars with a volume of 300 mL were partially filled with approximately hundred gram of sprouts from the inbred line TC130 and the cultivars Spr 110, Philemon, Corinth and Kundry and closed with a lid equipped with a sampling septum. The production of ethylene was determined in 1 mL headspace samples 24, 48 and 72 hr after the start of the experiment and expressed as $\mu L\ L^{-1}\ g^{-1}$ sprout mass.

Expression of LSL in Sprouts under the Influence of GA$_3$ and Zeatin

Sprouts from the inbred line TC130 and the cultivars Ajax and Corinth were harvested at the point of maturity, disinfected for 10 min in 1% (m/v) sodium hypochlorite and rinsed three times with sterile demineralized water. Four sprouts per inbred line or cultivar were placed in transparent polycarbonate jars with a lid (size:18*10*6 cm (length*width*height)) with their plant connection feet in 1% (w/v) plain water agar or 1% (w/v) water agar containing respectively 1 mM GA$_3$, 5 mg/L zeatin or a combination of 1 mM GA$_3$ and 5 mg/L zeatin and kept at 20 °C with a 16 hr photoperiod (10 klux) in a growth room. The expression of

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LSL was scored as the number of yellow wrapper leaves on the four sprouts 9 and 12 days after the start of the experiment and expressed as a percentage of the total number of wrapper leaves present on the sprouts.

Screening of Ethylene Insensitivity on Detached Leaves

Third leaves of 5 weeks old plants of the inbred line TC130 and the cultivars Corinth and Ajax were collected by means of cutting of the petiole with a scalpel close to the petiole-stem connection. Five leaves per inbred line or cultivar were put separately into 50 mL tubes filled with nutrient solution N1 containing 0 or 100 mg/L Ethrel. The nutrient solution N1 was comprised of 1 mM NH_4^+, 8 mM K^+, 3.2 mM Ca^{2+}, 1.6 mM Mg^{2+}, 14.4 mM NO_3^-, 1.6 mM SO_4^{2-}, 1 mM PO_4^{3-}, 15 μM Fe^{2+}, 7 μM Mn^{2+}, 20 μM B^+, 0.5 μM Cu^{2+} and 0.5 μM Mo^{2+} and adjusted to pH = 5.6. Nutrient solution consumed by the leaves was replenished daily. The leaves were kept at 20 ºC with a 16 hr photoperiod (10 klux) in a growth room and evaluated for the rate of yellowing for 7 days after the start of the experiment. Yellowing was scored as a percentage of the total leaf blade area.

The rate of yellowing was also monitored on the fifth leaf of plants at an age of 5 months. This experiment was essentially conducted as described for younger leaves but the evaluation of the yellowing rate was extended to a period of 4 weeks as the yellowing process in older leaves proceeded much slower than in younger leaves.

Induction of Senescence in Detached Leaves under the Influence of 2,4-D and HFCA

The fifth leaf of 5 months old plants from the inbred line TC130 and the broccoli cultivar Emperor (cultivar from Sakata, Japan) and the Brussels sprout cultivars Ajax and Corinth were used to study the influence of a concentration range of the auxin 2,4-D on the yellowing rate of detached leaves. Leaves (5 leaves per treatment per cultivar) were put with their petioles in 50 mL tubes filled with nutrient solution N1 containing 0.0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/L 2,4-D. Nutrient solution consumed by the leaves was replenished daily during the experiment. The leaves were evaluated daily for the rate of yellowing on the following 7

days. Yellowing was scored as a percentage of the total leaf blade area.

In a parallel experiment, the influence of polar auxin transport inhibition on the rate of yellowing was screened. Leaves (5 leaves per treatment per cultivar) from the inbred line TC130 and the broccoli cultivar Emperor were put with their petioles in 50 mL tubes filled with nutrient solution N1 containing 0 or 10 μM 9-hydroxy-fluorone carboxylic acid (HFCA), a polar transport inhibitor. HFCA was dissolved at a concentration of 10 mM in 1 M potassium hydroxide and diluted 10^5 times in the nutrient solution. The yellowing of the leaves was monitored for 2 weeks and scored as described previously.

Determination of the Cytokinin Content and Composition in Brussels Sprouts

The content and distribution of cytokinins in Brussels sprouts was determined in lyophilized Brussels sprout samples. Edible sprouts (approximately 200 g per inbred line or cultivar) from the inbred lines TC130 and DC69 and the cultivars Spr 105, Adonis, Tavernos and Ajax were lyophilized (Breda Scientific, The Netherlands) for 2 days and subsequently homogenized into a flour with an electric coffee mill (Philips, The Netherlands). The dry weight of the lyophilized samples was recorded afterwards and used for the calculation of the content of cytokinins per g fresh weight. The content of zeatin, DHZ and iP type cytokinins in the dry samples was determined according to Motyka et al. (1996) and expressed in pmol gfw^{-1}.

The Influence of Kinetin on the Germination and Development of Brussels Sprout Seedlings

Seeds from Brussels sprout inbred lines and cultivars were disinfected for 10 min in 1% (m/v) sodium hypochlorite and rinsed for 3 times with sterile demineralized water prior to sowing. Twenty seeds per cultivar were sown in 20*10*5 cm (length*width*height) white polycarbonate boxes with a transparent lid on plain 1% (w/v) water agar containing 0 and 15 mg/L kinetin. The seeds were allowed to germinate in darkness for three days at 20 °C, and subsequently exposed to conventional TL light-tubing (Philips, 10.000 lux) with a 16-h photoperiod at 20 °C. Seedlings were evaluated after 2 weeks for developmental

parameters such as germination, root development, hypocotyl elongation and cotyl expansion.

The Influence of Papaverin on Germination and Development of Brussels Sprout Seedlings

Seeds from Brussels sprout inbred lines and cultivars were disinfected for 10 min in 1% (m/v) sodium hypochlorite and rinsed for 3 times with sterile demineralized water prior to sowing. Twenty seeds per cultivar were sown in 20*10*5 cm (length*width*height) white polycarbonate boxes with a transparent lid on plain 1% (w/v) water agar containing 0 and 5 mM papaverin, an inhibitor of $N^{7,9}$-glucosylation of cytokinins (Tao et al., 1991). The seeds were allowed to germinate in darkness for three days at 20 °C, and subsequently exposed to conventional TL light-tubing (Philips, 10.000 lux) with a 16-h photoperiod at 20 °C. The performance of the inbred lines and cultivars was evaluated one week after sowing for germination percentage and seedling development. Seedling development on water agar in the presence and absence of papaverin was scored as percentage of seedlings with root protrusion, hypocotyl elongation and cotyl expansion.

Measurement of Polar Auxin Transport in Brussels Sprout Seedlings by means of their Gravitropic Response

Seeds from Brussels sprout inbred lines and cultivars were germinated and grown on plain 1% (w/v) water agar containing 0 and 10 μM HFCA according to the protocol of the previous paragraph. Seedling development on 0 and 10 μM HFCA was evaluated 1 week after germination with special attention for typical auxin transport related aspects such as lateral root formation, hypocotyl elongation and gravitropic responses. The gravitropic response of seedlings was used as a marker for polar auxin transport in the hypocotyl and measured as the average angle (in °) between seedling hypocotyls and the horizontal. Seedlings with an angle of 90° with respect to the horizontal on 10 μM HFCA have a high capacity of polar auxin transport, seedlings with a small angle (e.g. 20°) have a low capacity.

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Chapter 8
General discussion

The vegetable business chain can start to market tasty Brussels sprouts.

The vegetable business has in the past decade quickly evolved from a production-driven - into a specialised organisation with tailor-made products for various types of consumers. Most of the vegetables are currently sold by retailers many of which want continuity of supply, exact harvest planning, environmentally friendly production and innovation with respect to aspects such as nutritional value, flavour, shelf life and presentation. Most of the VBC's desires concerning quality and performance related traits are met in the current cultivars of vegetable crops. Breeding companies have in general optimised most crops for traits such as yield, uniformity, reliability and field performance. Growers can produce most of the vegetables in an environmental friendly way due to advanced developments in the field of biological pest control and the introduction of natural resistances against major diseases which previously could only be controlled with frequent fungicide or pesticide sprays. The most important aspects of vegetable quality for consumers: taste, health and nutritional value, are, however, hardly covered by the members of the VBC. An increasing number of consumers are demanding vegetables with a constant quality for these traits also, but usually cannot buy them on a regular basis from the current retailers. The lack of vegetables with reliable expression of intrinsic quality traits on the shelves of vegetable departments of retailers and vegetable stores has many causes which have already been discussed in the introduction of this thesis (see Chapter 1). The overriding reasons are, however, poor definition of consumer preferences in vegetable crops, a lack of convenient tools for the determination of the key intrinsic quality traits related to consumer preference and poor definition of intrinsic quality traits for cultivars of vegetable crops.

In this thesis, probably the first practical example in the history of plant breeding is given of an integrated approach of the definition of consumer preference for Brussels sprouts, the development of methods for the measurement of preference determining compounds, the application of these methods for the breeding of tasty cultivars and the definition of environmental

parameters which influence the content of consumer preference related compounds.

Large scale analytical assays are essential for a successful approach.

The development of Brussels sprouts with improved consumer quality was started by Novartis Seeds approximately 12 years ago, in a period when most of their cultivars combined a high agronomic performance with a poor taste. The early work of Fenwick and co-workers (1983), on the role of glucosinolates in the development of bitterness in Brussels sprouts, showed the convincing evidence that the poor taste of the commercial assortment of cultivars was related to a high content of bitter glucosinolates. No data were, however, available to confirm our hypothesis, since the complaints from the market were not supported by reliable consumer preference scores and the content and distribution of glucosinolates in cultivars with a supposed poor flavour had not been determined.

It was decided that, from then on, only cultivars with an improved flavour compared to the current assortment could be introduced. Such a policy can only be successful when the key factors for consumer preference of Brussels sprouts are defined, generally accepted, and measurement is feasible in a large breeding program. This long-term project for the improvement of sprout flavour was the most important reason to start the development of assays for the large scale determination of sinigrin and progoitrin content of Brussels sprouts in the first place. The success of the mission was strictly dependent on the availability of the former assays. Chapters 2 and 3 describe the successful development of novel large scale assays for glucosinolates and form the basis of this thesis. These assays have particularly been used to study the relationships between glucosinolates and consumer preference (Chapter 4), the inheritance of the flavour-determining glucosinolates sinigrin and progoitrin (Chapter 5), and the influence of root architecture and environmental parameters on the content of sinigrin and progoitrin (Chapter 6).

It would not have been possible to conduct these large scale studies with the old conventional methods for the determination

of glucosinolates as described in the introduction of Chapter 2. The specific polyclonal ELISA assays for the glucosinolates sinigrin and progoitrin (Chapter 2) and the assay for the direct determination of glucosinolates after degradation of endogenous glucose (Chapter 3) have revolutionised the screening of the glucosinolate content and distribution in the Novartis Seeds breeding program for Brussels sprouts. The ELISA assays for sinigrin and progoitrin are clear examples of polyclonal antisera with the specificity of monoclonals, which are usually preferred for scientific purposes (e.g. Kreuger and van Holst, 1995).

The assays have, in the first place, been used to determine the extent of variation in the content of flavour-affecting glucosi-nolates and subsequently for the efficient definition of consumer preference. In our experience the successful determination of relationships between the content of flavour-affecting compounds and consumer preference scores depends for the main part on sufficient variation in the compounds of interest in the samples offered to a consumer panel. In our situation, the preparation of a successful consumer trial always starts with the development of large scale assays for compounds suspected of affecting flavour and the screening of the germplasm for variation in these compounds. The role of the glucosinolates sinigrin and progoitrin in consumer taste preference for sprouts was defined using Brussels sprouts samples containing the maximum variation in these compounds, while no relationships could be observed at glucosinolate levels lower than the complaint-eliciting limit (Chapter 4). In a vast number of papers the relationships between flavour related compounds and consumer preference are defined using complex, small-scale analytical methods which, however, often cannot be translated into convenient assays for the efficient screening of these compounds in breeding programs (see Chapter 1). Breeding for improved intrinsic quality in vegetables is only efficient when, according to our comments in Chapter 1, time consuming expensive analytical methods for the determination of flavour-related compounds can be translated into large scale relatively cheap assays which allow their measurement in extensive breeding programs.

Consumer preference studies form the key to the successful breeding and marketing of vegetables with improved intrinsic quality.

The success of many consumer products is based on their continuous availability and their constant quality in terms of appearance, flavour, texture and packaging. Vegetables are typical examples of heterogeneous consumer products for aspects such as appearance, flavour (Chapters 4 and 5), shelf life (Chapter 7), texture and in many cases are not available year-round. As stated in Chapter 1 of this thesis, consumers in general do not have an universal appreciation for vegetable intrinsic quality aspects such as flavour and tend to differentiate vegetables from specific crops in segments with respect to flavour related characteristics such as sourness, sweetness, bitterness etc. Brussels sprouts are a good example of a vegetable which creates mixed feelings in the consumer world because of its pronounced flavour based on the presence of glucosinolates. Currently, about half of the total volume of Brussels sprouts in the Netherlands is consumed by 25% of the Brussels sprout consuming population. Many people do not consume Brussels sprouts at all because of their typical (bitter) flavour. Critical or reluctant consumers only can be attracted to buy Brussels sprouts when the risk of purchasing bitter sprouts is zero. In other words, there is a need for reliable non-bitter sprouts in the market and for establishment of the glucosinolate content in sprouts related to the no-complaint level.

Chapter 4 of this thesis describes the consumer preference studies conducted in the past decade to define the consumers preferences for sprouts and the role of glucosinolates in flavour and bitterness of sprouts. These studies clearly revealed that even regular Brussels sprout consumers dislike too much sinigrin and progoitrin, two glucosinolates with a bitter taste as an intact glucosinolate, or after enzymatical degradation (Fenwick et al., 1983). Consumer preference for Brussels sprouts was, relatively speaking, not correlated to a complex array but only to two flavour determining compounds, and this allowed efficient breeding for improved flavour (Chapter 5).

Our consumer and expert taste trials have learnt us that regular consumers do not differentiate Brussels sprouts for

flavour-related aspects, but segmentation of Brussels sprouts into two groups with different bitterness levels might attract a new group of Brussels sprout consumers. Regular Brussels sprouts consumers should be supplied with sprouts containing less than 2.2 g kg^{-1}, candidate consumers preferably have to be attracted with sprouts containing less than 0.6 g kg^{-1}. Expert panels can still differentiate Brussels sprouts samples for bitterness below a sinigrin and progoitrin sum of 2.2 g kg^{-1} and observe a correlation between bitterness and the sum of sinigrin and progoitrin between 0.5 and 2.2 g kg^{-1}. Consumers of Brussels sprouts in the UK use the term supersweet for sprouts with a glucosinolate content below approximately 0.6 g kg^{-1}. This low glucosinolate content might be the ideal level to attract consumers who currently dislike Brussels sprouts because of their potential bitterness.

In the last 10 years, the selection pressure for a low sinigrin and progoitrin content has resulted in new cultivars with an average sum of sinigrin and progoitrin below the 0.6 g kg^{-1} limit. Critics might fear that reduction of the sinigrin and progoitrin content in Brussels sprouts will lead to a flavourless product and complaints from consumers with a preference for bitterness. From the remarks of consumers, which have been recorded during the various taste trials, it was, however, clear that bitterness-appreciating consumers also liked non-bitter sprouts, suggesting that a general decrease in the sinigrin and progoitrin will not lead to complaints from the current consumers of Brussels sprouts.

High heritability values allowed the quick reduction of the sinigrin and progoitrin content in Brussels sprouts and resulted in low aliphatic glucososinolate contents.

After the establishment of the relationships between the sinigrin and progoitrin content and consumer preference and the definition of the critical level of these glucosinolates for regular consumers a large scale breeding program for optimal flavour in Brussels sprouts was initiated. At that time, only limited information was available with respect to the inheritance of the sinigrin and progoitrin content in Brussels sprouts from the group of Fenwick and co-workers (Heaney and Fenwick, 1980a and b; Fenwick et al., 1983). The application of the ELISA assays

(Chapter 2) and the glucosinolate assay (Chapter 3) facilitated the screening of the sinigrin, progoitrin and sum of glucosinolates in a large series of F1-hybrids and their corresponding parental lines. The screenings demonstrated high broad - and narrow-sense heritability values for all glucosinolates and relatively straight forward inheritance of glucosinolates in Brussels sprouts (Chapters 3 and 5). The sinigrin, progoitrin and sum of glucosinolates was determined for approximately 60% by the genetic background of the cultivars and for 40% by environmental conditions, allowing a quick reduction in the content of both bitter glucosinolates in successive selection cycles. The observed results contrasted with the expectation that a seven step (sinigrin) or nine step (progoitrin) biosynthesis route would not inherit in a simple intermediate way (see Introduction of Chapter 5). A detailed analysis of all glucosinolate profiles gathered in F1-hybrids and their corresponding parental lines supplied essential information about the biosynthesis route of aliphatic glucosinolates in Brussels sprouts and possible ways to manipulate the distribution of aliphatic glucosinolates in this crop.

The results of Chapter 5 indicate, in analogy to the findings of Magrath et al. (1994), an important role for the elongation enzymes involved in the extension of methionine into the precursors of respectively C_3, C_4 and C_5 aliphatic glucosinolates and the introduction of these precursors in the side-chain modification routes by means of mixed-function monooxygenases (MFOs) (Bennett et al., 1996). Sinigrin and progoitrin are end-products of the side chain modification routes of C_3 and C_4 glucosinolates (see Figure 5 of Chapter 5) and can only be abundant in the previous commercial generation of our F1-hybrids when the former enzymes have a high activity. The elongation enzymes and MFOs, in collaboration, determine the cumulative content of C_3, C_4 and C_5 aliphatic glucosinolates, but have no influence on the distribution of particular glucosinolates within these elongation groups. Particular glucosinolate desaturases and glucosinolate hydroxylases, which, respectively, catalyse the synthesis of alkenyl-glucosinolates from methylsulphinylalkyl glucosinolates and the addition of a hydroxyl-group to alkenyl

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glucosinolates (Mithen et al., 1995), are apparently also quite active in these cultivars.

In our attempts to improve Brussels sprouts flavour, by decreasing the content of sinigrin and progoitrin, the sum of glucosinolates declined simultaneously during the selection process and was highly correlated with the sinigrin and progoitrin content in all cultivars developed (Chapter 5, Figure 4; Chapter 3, Figure 5). These results tell us two things. Firstly, the selection pressure for a low sinigrin and progoitrin content seems to have resulted in parental lines and F1-hybrids with a low introduction rate of elongated methionine precursors for C_3 and C_4 glucosinolates due to a low activity of elongation enzymes and/or MFOs. Secondly, the enzymes in the side chain modification routes for sinigrin and progoitrin are not affected in "low" parental lines and F1-hybrids, since the proportion of sinigrin and progoitrin in the sum of glucosinolates generally remains the same during the selection process (Chapter 3, Figure 5). The improvement of flavour in Brussels sprouts by selection for a low sinigrin and progoitrin content has proved to be an efficient approach in terms of timing, heritability and acceptance of new cultivars by consumers.

Intermezzo: Breeding for optimal flavour and health-promoting activity in Brussels sprouts: The next step towards improved intrinsic quality. In the last decade, the potent activity of glucosinolate degradation products against various forms of cancer has continuously been highlighted (Johnson et al., 1994; Zhang et al., 1992) with special attention to glucoiberin (methylsulphinylpropyl glucosinolate) (Williamson et al., 1998) and glucoraphanin (methylsulphinylbutyl glucosinolate) (Faulkner et al., 1998), non-bitter precursors of, respectively, sinigrin and progoitrin in the C_3 and C_4 side chain modification routes. Recently, it was demonstrated that the sinigrin degradation product allyl isothiocyanate stimulated programmed cell death (apoptosis) in the colon of dimethylhydrazine treated rats thus selectively destroying pre-cancerous cells in the colonic mucosa (Smith et al., 1998). The former findings provided arguments for a balanced glucosinolate distribution in Brussels sprouts to ensure both optimal flavour and health-promoting activity. A breeding approach in which sinigrin is partially replaced by glucoiberin, and progoitrin by glucoraphanin, is preferable to a general decline in C_3 and C_4 glucosinolates and will result in non-bitter health-promoting Brussels sprouts. New parental lines with a high content of glucoiberin and glucoraphanin are required as genitors for the breeding of new tasty, health-promoting Brussels sprouts cultivars. Such lines can in principle be screened with specific ELISA assays for the former glucosinolates, analogous to the determination of sinigrin and progoitrin in Chapter 2. The development of tasty and health-promoting Brussels sprouts cultivars requires a comparable approach to that described for the development of tasty cultivars in Chapters 2 to 6 in this thesis, but this time with the incorporation of the glucosinolates sinigrin, progoitrin, gluconapin, glucoiberin and glucoraphanin. Figure 1 depicts the hypothesised biosynthesis routes for aliphatic glucosinolates in Brussels sprouts in detail. The biosynthesis route of aliphatic glucosinolates in Brussels sprouts comprises, according to Chapter 5, three parallel side chain modification branches for C_3, C_4 and C_5 glucosinolates, each of them being fed with, respectively, elongated C_3, C_4 and C_5 methionine precursors.

In figure 1 the methionine elongation pathway is presented in yellow, the C_3 pathway in mintgreen, the C_4 pathway in blue, and the C_5 pathway in purple. A selective increase in the content of glucoiberin and glucoraphanin in Brussels sprouts, at the expense of, respectively, sinigrin and progoitrin, seems on the one hand to be possible by reduction of the activity of the desaturase enzymes which catalyse the splicing of the methylsulphinyl-group from the former glucosinolates and on the other hand by a high activity of the enzymes involved in the production of C_3 and C_4 precursors for the biosynthesis of C_3 and C_4 glucosinolates (see previous paragraph). The green bars adjacent to the elongation enzymes ELN1 and ELN2 and the mixed function monooxygenases FMO1 and FMO2 in figure 1 highlight which enzymatic reactions are expected to stimulate accumulation of glucoiberin and glucoraphanin in Brussels sprouts. Suppression (red bar adjacent to ELN3) of the elongation enzyme ELN3 should prevent the conversion of dihomomethionine, the precursor of C_4 glucosinolates, into trihomomethionine.

The modifications in the side chains of the various glucosinolates, as shown in the lower half of figure 1, take place following a distinct pattern. Oxidation of the thiomethyl-group of glucosinolates by the enzyme thiomethyl sulphoxidase (TMSO), is followed by the removal of the sulphinylmethyl-group by the enzyme desaturase (DS) to yield glucosinolates with an unsaturated side chain (Giamoustaris and Mithen, 1996), or a second oxidation step by a currently not characterised enzyme sulphinylmethyl sulphoxidase (SMSO) to yield glucosinolates with a sulphonylmethyl-group. Removal of the methylsulphinyl-groups from glucoiberin and glucoraphanin yields, respectively, sinigrin and gluconapin. The desaturation or further oxidation of methylsulphinyl-groups should be prevented for a proper accumulation of glucoiberin and glucoraphanin. The red bars adjacent to the desaturases DS1 and DS2 and the sulphinylmethyl sulphoxidase SMSO in figure 1 show which enzymatic conversions should be prevented for the accumulation of the former glucosinolates.
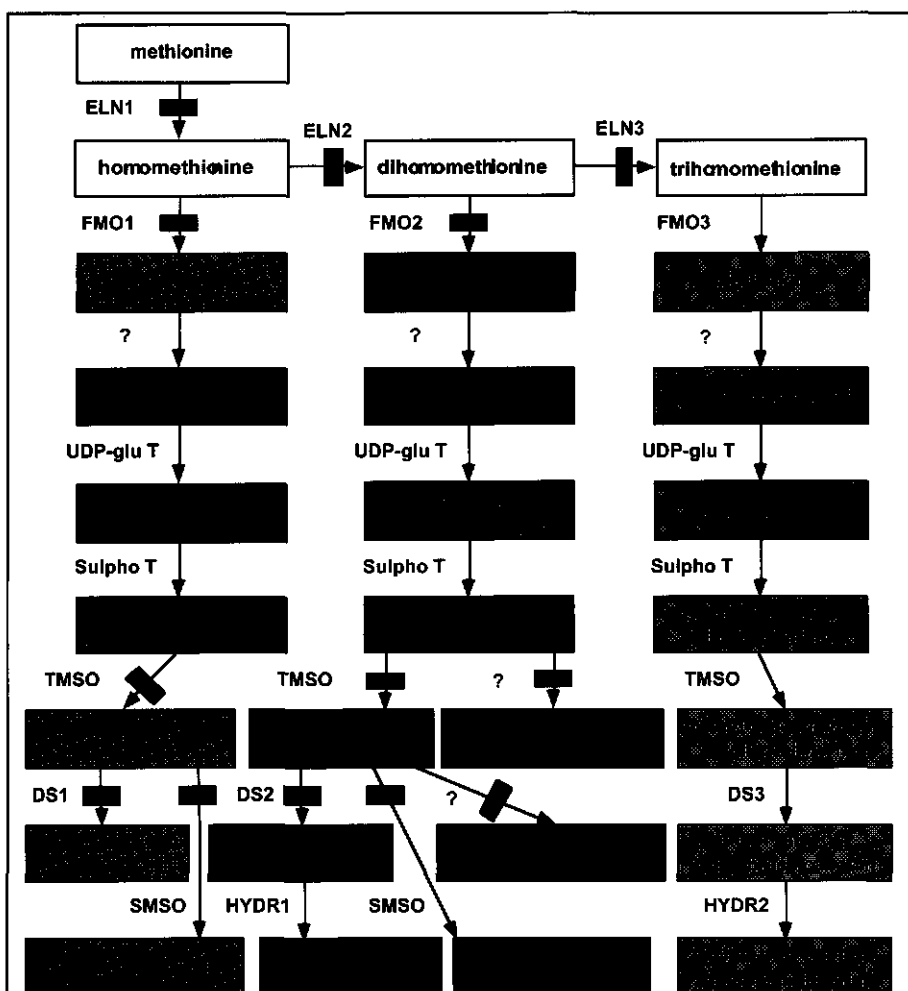
Figure 1. Model for the biosynthesis of methionine derived aliphatic glucosinolates in *Brassica* vegetables. The biosynthesis route of aliphatic glucosinolates comprises three parallel side chain modification branches for C_3, C_4 and C_5 glucosinolates, each of them being fed with, respectively, elongated C_3, C_4 and C_5 methionine precursors. The elongation pathway is depicted in yellow, the C_3 pathway in mintgreen, the C_4 pathway in blue, and the C_5 pathway in purple. Abbreviations: ELN1-3 = methionine elongation enzyme 1-3, FMO1-3 = mixed function monooxygenase 1-3, UDP-glu T = UDP-glucose:thiohydroximate glucosyltransferase, Sulpho T = 3'PAPS-5'-phosphosulphate:desulphoglucosinolate sulphotransferase, gs = glucosinolate, TMSO = thiomethylsulphoxidase, DS1-3 = methylsulphinyl desaturase 1-3, SMSO = sulphinylmethyl sulphoxidase, HYDR1,2 = alkenyl hydrolase 1,2. The trivial names of the glucosinolates are given between brackets. Red bars highlight the enzymatic conversions which should be inhibited to get accumulation of glucoiberin and glucoraphanin. The green bars depict which enzymatic steps need to be stimulated for the accumulation of the former glucosinolates.

The conversion of glucoerucin and glucoraphanin into respectively glucoraphasatin and glucoraphenin are alternative options to prevent the accumulation of glucoiberin and glucoraphanin (see red bars adjacent to the question marks). These enzymatic conversions seem however to be exclusively present in edible parts of the genus *Raphanus sativus.* The unsaturated side chain of glucosinolates such as gluconapin can be hydroxylated by the enzyme hydroxylase (HYDR) to yield hydroxylated glucosinolates such as progoitrin (Parkin et al., 1994). The enzymatic degradation products of sinigrin and gluconapin display 30 to 50 times less bioactivity than those of glucoiberin and glucoraphanin in bioassays using cultured human cells in vitro (Williamson et al., 1998). The disparate distribution of glucoiberin, glucoraphanin and glucoallysin within, respectively, the groups of C_3, C_4 and C_5 glucosinolates in Brussels sprouts (Table 2, Chapter 5; Heaney and Fenwick (1980) and Carlson et al. (1987) for the distribution of C_3 and C_4 glucosinolates) suggests the occurrence of different DS enzymes with a high specificity towards the sulphinilated C_3, C_4 and C_5 side chains of these glucosinolates. The presence of alleles for highly specific desaturase enzymes in Brussels sprouts germplasm allows the breeding of tasty health-promoting cultivars with an optimum distribution of sinigrin, glucoiberin, glucoraphanin and progoitrin. Genitors with a high production of aliphatic glucosinolates have been characterized in Chapters 3 and 5, which can serve as starting material for the breeding of cultivars with a high and balanced profile of tasty and healthy glucosinolates. Enhancement of the content of glucoiberin and glucoraphanin will automatically result in lower contents of sinigrin and gluconapin and consequently to low levels of progoitrin.

The right cultivar choice and control over glucosinolate content affecting environmental parameters results in predictable contents of glucosinolates and optimal flavour for consumers.

The results of Chapters 3 and 5 demonstrate that the content of sinigrin and progoitrin and the sum of glucosinolates is determined for approximately 60% by phenotype and for 40% by environmental parameters. Ten years ago, consumers of sprouts were confronted with a collection of commercial Brussels sprouts cultivars containing a sum of sinigrin and progoitrin between 2.5 and 7.7 g kg^{-1}, which were judged on average between moderate and very bitter. At that time, the majority of the variance in glucosinolate content and flavour between sprout samples was related to the cultivar choice, and all cultivars had a sum of sinigrin and progoitrin above the consumer acceptance limit of 2.2 g kg^{-1} (Chapter 4).

Ten years of selection pressure for a low sinigrin and progoitrin content has resulted in a new series of Brussels sprout cultivars with a low sum of glucosinolates and an optimum flavour. Currently, consumers have an assortment of tasty sprouts with a low sum of sinigrin and progoitrin (e.g. trial 3 from Table 2 of Chapter 4). The majority of the variation in glucosinolate content and flavour is now related to the influence of environmental parameters. In Chapter 6 the influence of environmental parameters on the content of sinigrin and progoitrin of Brussels sprout cultivars is described. The sum of sinigrin and progoitrin of cultivars, and variation in the sum of the former glucosinolates under the influence of environmental parameters, is determined by the genetics of root architecture. Root architecture parameters such as tap root length, lateral root length and distribution of lateral roots along the tap root determine the glucosinolate content and its deviations in different environments. Rainfall and the soil sulphate concentration are identified as the key environmental parameters which, in collaboration with the root system, determine the sinigrin and progoitrin content of cultivars. The current Brussels sprout cultivars will not accumulate a glucosinolate content

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Titel: Gevonden voorwerp (Umberto, 1993)
Afmeting: Inclusief lijst 45 x 35 cm
Techniek: Gemengde techniek
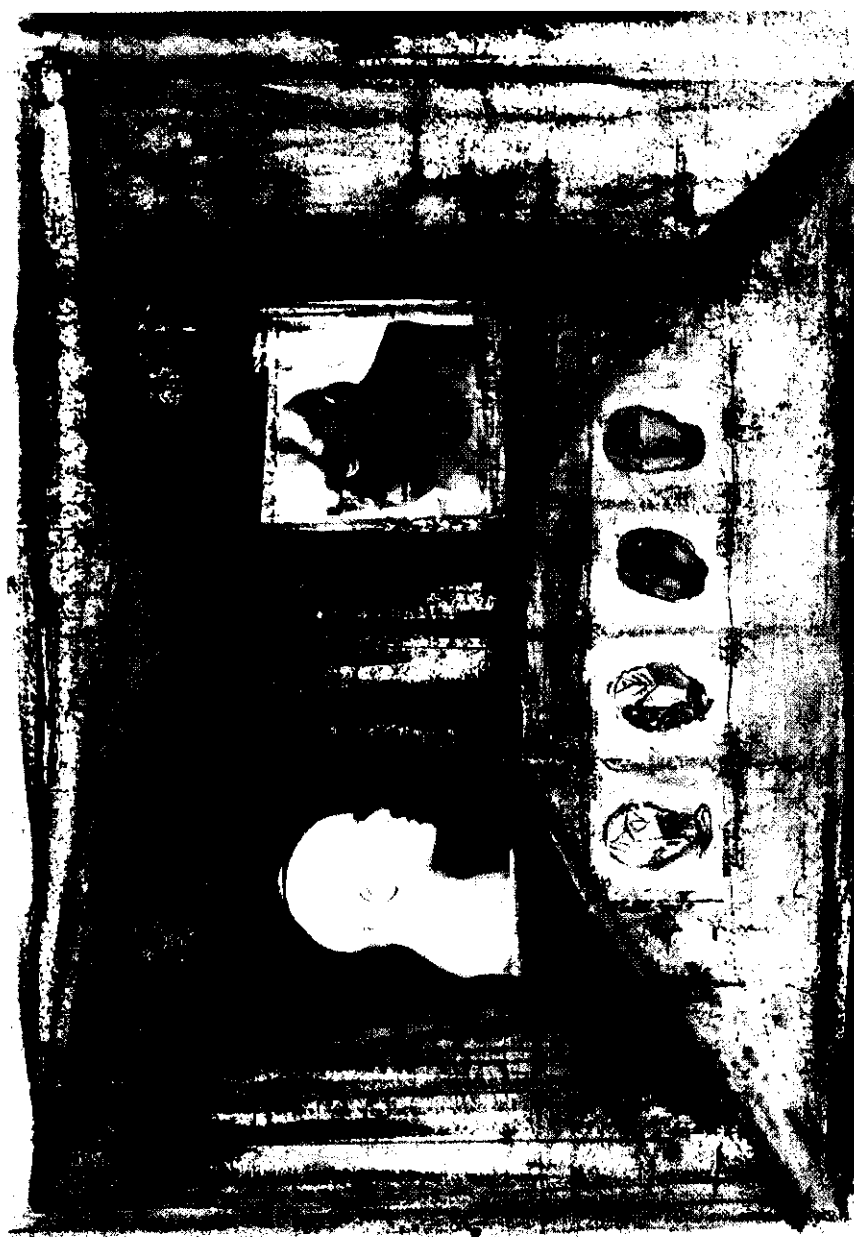
Titel: Evolutie-leer (Umberto). Afmeting: Inclusief lijst 81 x 61 cm. Techniek: Gemengde techniek op aquarel papier.

above the 2.2 g kg^{-1} level when grown in soils with a sulphur content of 50 kg ha^{-1} or less at planting time.

The availability of Brussels sprouts cultivars with an optimum flavour in all earliness segments and simple tools for soil sulphur content management make it possible to produce sprouts with a reliable glucosinolate content and thus flavour for consumers. Currently, retailers and other members of the VBC can control the flavour of Brussels sprouts by means of cultivar choice and cultivation instructions with respect to sulphur management thus guaranteeing sprouts with an optimum flavour for consumers.

The added value of long shelf life for the maintenance of intrinsic quality of sprouts.

The glucosinolate content of Brussels sprouts cultivars can accurately be predicted at the point of optimum maturity using the knowledge in this thesis. The results of Chapter 3 demonstrate, however, that the glucosinolate content in attached sprouts of cultivars is highest at the point of optimum maturity and declines steadily in time (see Figure 3 of Chapter 3), presumably due to senescence-driven degradation or reallocation of glucosinolates. The glucosinolate content of picked sprouts declines in time with a degradation rate of approximately 10% per day, demonstrating the need for the preparation of glucosinolate samples directly after harvesting (recent observations). The decline of glucosinolates in picked sprouts coincides with the senescence process suggesting a causal relationship between deterioration of sprouts and as a consequence the enzymatic degradation of glucosinolates. The degradation of glucosinolates is catalyzed by a family of thioglucosidase enzymes, better known as myrosinases (Bones, 1990; Thangstad et al., 1990; Bones and Iversen, 1985; Phelan et al., 1984, Iversen and Baggerud, 1980), which are located in plant tissues separated from their substrates. Tissue disruption, e.g. grazing, leads to contact between glucosinolates and thioglucosidase and their degradation into glucose, sulphate and their corresponding isothiocyanates, thiocyanates and nitriles (Uda and Maeda, 1986; Springett and Adams, 1988).

Although direct evidence is lacking, these observations suggest that the senescence process disrupts the integrity of

cells and brings together thioglucosidase and glucosinolates thus facilitating the degradation of glucosinolates. Indirect evidence shows that in cruciferous plants periods of biosynthesis are followed by periods of degradation of glucosinolates (McGregor, 1988; Clossais-Besnard and Larher, 1991; Rosa et al., 1994 and 1996). More evidence for the degradation of glucosinolates in intact plants is given by Spence and Tucknott (1983), who showed that degradation products from glucosinolates were accumulated in the epicuticular wax of watercress, a glucosinolate containing crucifer.

During our consumer taste trials (Chapter 4, table 2), the frequency of remarks on bitterness was much higher in the January trials of 1991 and 1995 compared to the November trial of 1993, presumably due to the age-dependent degradation of glucosinolates in the sprouts of the former trials.

The long shelf life (LSL) trait, as described in Chapter 7, delays senescence by more than two weeks at room temperature and will give the storability of Brussels sprouts in the VBC a new dimension in terms of maintenance of a fresh green appearance and attractivity for consumers. The LSL trait also will greatly influence the intrinsic quality of sprouts by maintaining the glucosinolate content in the post harvest period. The LSL trait seems to be ideal to ensure the quality of sprouts in terms of fresh appearance, flavour and the content of health-promoting glucosinolates.

Future needs for the marketing of Brussels sprouts with health and flavour claims.

Currently, most vegetables are offered to consumers with information regarding the quality class, country of origin and, in the case of fruity vegetables, often the name of the growers-co-operative responsible for their production. Information regarding flavour, texture and content of healthy compounds is on average poorly communicated by means of general labelling or suggestive branding. The enormous number of suggestive brands, for e.g. tomato, in general cause more confusion than clarity with respect to the communication of relevant product information to consumers. The brands are in fact only useful as a description of

Titel: Proud to be a sprout (Umberto, 1996)
Afmeting: Exclusief lijst 70 x 62 cm
Techniek: Gemengde techniek op papier

Titel: De spruiteters (Umberto). Afmeting: 93 x 92 cm. Techniek: Kleurenreproductie.

segments of vegetable producers in the VBC, but not for the differentiation of the produce for quality related aspects.

The lack of informative labels with respect to intrinsic quality related parameters can be explained by a couple of reasons. Firstly, the old style auction system until recently created large blocks of vegetables with a homogeneous composition for visible intrinsic quality aspects such as colour, size, firmness and supposed shelf life but not for invisible intrinsic quality related aspects such as flavour, texture and the content of health promoting compounds. Vegetables in such blocks have lost their identity in terms of cultivar name, and with that, the information with respect to the expression of cultivar-dependent intrinsic quality traits (this thesis). Secondly, but even more relevant, the members of the VBC do not have accurate methods for the assessment of intrinsic quality aspects of vegetables and even lack reliable consumer preference data for most crops.

Brussels sprouts are the first crop for which all essential information with respect to consumer preference mapping, measurement of key flavour determining compounds and the influence of phenotype and environment is available for use in quality control in the VBC. The results of this thesis allow the production of Brussels sprouts with a predictable glucosinolate content and flavour, thus opening ways for the reliable labelling of flavour claims. The process of quality control should be based on two criteria: Cultivar choice and soil sulphur management. In the ideal situation quality control should be based on measurement of sinigrin and progoitrin, the compounds responsible for flavour of Brussels sprouts. Our current large scale methods are still not accurate enough to apply them for flavour control in the VBC. Members of the VBC need fast, non-destructive and reliable measurements for the determination of sinigrin and progoitrin in Brussels sprout samples for an efficient flavour assessment. Recent technical developments, have resulted in novel methods for the purpose of quality control according to the former criteria. The sum of glucosinolates and individual glucosinolates in seeds of various *Brassicas* can be determined by Near Infra Red Spectroscopy (NIRS) in a range between 6 and 193 μmol g^{-1} seed, concentrations which correspond with 0.3 to 9.0 g

glucosinolates 100g^{-1} seed (Velasco and Becker, 1998). NIRS seem to be very attractive for the determination of the glucosinolate content and profile of Brussels sprouts, but the concentration range of glucosinolates in tasty sprouts is below 0.3 g 100g^{-1}, and might be the limiting factor for its application. Should the glucosinolates in sprouts as in white cabbage (Pocock et al., 1987) be distributed gradient-wise from a medium content in the core to a high content in the outer leaves, the detection limit will not be the limiting factor for their determination with NIRS technology.

The successful marketing of Brussels sprouts, and derived products, with an enhanced content of health-promoting glucosinolates will be dependent on reliable health claims, since the legislation controlling the labelling of health claims and the content of health promoting compounds requires this. Reliable health claims with respect to the health-promoting activity of specific glucosinolates have to be substantiated in large scale human intervention studies.

Application of intrinsic quality knowledge in the vegetable business chain.

In this thesis, Brussels sprouts cultivars have been developed using an integrated approach with an optimal field performance and flavour for consumers based on a low content of the glucosinolates sinigrin and progoitrin. Precise sulphur fertiliser management in combination with the right cultivar choice will guarantee the production of tasty sprouts for all members of the VBC. The development of tasty, productive Brussels sprouts by breeding companies is the first requirement to be met for the successful marketing of tasty sprouts by members of the VBC. The reliable availability of tasty cultivars throughout the whole season and clear instructions with respect to sulphur management should be an ideal starting point for retailers to start the branding of flavour in Brussels sprouts. Branding of Brussels sprouts for low, intermediate and pronounced bitterness will lead to a situation in which consumers can purchase their preferred flavour regularly and reliably. The prerequisites for the marketing of tasty sprouts have been met by our breeding company, the marketing itself is a task for the other members of

the VBC and hopefully will be implemented on a large scale from now on. Flavour of Brussels sprouts is a clear example of a high priority trait in which a quick improvement was needed for the short term survival of the crop in the VBC. The marketing and branding of tasty sprouts is, therefore, an ideal test case to see whether products with improved intrinsic quality can gain a place in the market. The successful marketing and branding of tasty sprouts might speed up the development of other intrinsic quality traits in other crops, lack of interest might slow down new developments in the field of intrinsic vegetable quality. Whatever happens to the productive, tasty Brussels sprouts cultivars in the market, this thesis has proven that breeding for improved intrinsic quality in vegetables is feasible in a reasonable period of time.

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Brussels sprouts
Inspiration for science and art

About ten years ago, at the start of our program for improved flavour, Brussels sprouts had a civil image and were associated with a poor taste and cooking smell by consumers. In the last decade Brussels sprouts have been moved from the shade into the spotlight and are transformed from an old-fashioned - to a modern vegetable. Currently, sprouts are healthy, tasty and, in a country such as the UK, almost year-round available in convenient packages.

The consecutive steps in the program for improved flavour, and the emotions we have experienced during the mission, have strikingly been painted by the Dutch artist Umberto. Umberto also was inspired by Brussels sprouts, but in an artistic sense. The contents of this thesis and Umberto's art give Brussels sprouts an upgrading in terms of consumer quality and artistry.

My team has developed the methods and created the knowledge for the production of fresh and tasty sprouts for growers and given them a better image for ordinary consumers. For Umberto, sprouts are synonymous with playfulness, humour, irony and parody and used in his art as a response to the intellectual art of the last decades. Umberto is producing ordinary art for ordinary people using the ordinary sprout as inspiration. One of my eleven statements "The art of breeding Brussels sprouts is inspiring Brussels sprouts art" is directly applicable to him. On the cover of this thesis and the general discussion a compilation of five of his paintings is used to describe the creation process of my thesis in terms of art. The position of the sprout in the present society is perfectly represented in his painting "Het levensverhaal (Lifetime story)" on the cover of my thesis. Sprouts are, along with bread, fish and love, his symbols for the key principles of life. The skull and bow are, according to me, symbols for our scientific contribution to the central position of sprouts in Umberto's world. The five sprouts on top of the painting symbolize the optimisation of flavour in sprouts. The sprout outline on the left is a representation of the poor-tasting cultivars at the start of the project which were steadily replaced

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by better ones through the years, as represented by the other sprouts in the center and the nice green sprout on the right.

The development of tasty sprouts has been a difficult job in terms of assay development, breeding for improved taste and the acceptation of our results in scientific papers and the vegetable business chain. The painting "Gevonden voorwerp (Found object)" perfectly describes the former process. The development of tasty sprouts is like uphill work: it is a time-consuming energy-demanding process with an unpredictable end. Only strong and inventive mountaineers survive.

The development of a new generation of tasty Brussels sprouts cultivars by technology-driven breeding can be regarded as an accelerated evolution process with the support of scientific selection instruments. His painting "Evolutie-leer (Evolution-theory)" symbolizes the breeding of tasty sprouts, analogous to the evolution of the Neanderthaler into the Homo Sapiens. The evolution of mankind took millions of years, the development of tasty sprouts fortunately a lot less.

The current sprouts combine agronomic traits such as nice appearance, shelf life and good flavour. Umberto's painting "Proud to be a sprout" symbolizes the new status of Brussels sprouts. Sprouts have evolved from a boring - into attractive vegetable with a future. The new sprouts cultivars in principle are attractive for most consumers and apparently also for artists like Umberto. The painting "De spruiteters (Sprout-eaters)" shows an average modern family during dinner eating tasty sprouts. Umberto and I hope that his art and the newly developed tasty Brussels sprouts will keep sprouts in the spotlight, the right place for a tasty, healthy and artistic vegetable.

Curriculum Vitae of Umberto

Umberto was born as Lambertus Joseph Augustinus Lemmens on 27th of October in Blerick, the province Limburg, The Netherlands. After a completed study Mechanics at the HTS in Heerlen (1979) his interest shifted to modern art in the beginning of the eighties. Umberto studied Industrial Design at the "Stedelijke Academie voor Schone Kunsten" in Hasselt Belgium (1986, cum laude). His

creations, projects and art have been exposed on many occasions from 1989 onwards. Expositions and media projects have been realised in Maastricht, Kassel, Berlin, Geleen, Venlo, Amsterdam, Bergamo, Antwerpen, Sittard and Hasselt. In the beginning of the nineties he started his one-man company Lemmens-i-Design+Art. He is a self-taught painter. In 1998 Umberto contributed to the international manifestation "Romanzero" in Sittard with his project "I am the inventor of round games and riddles and go to New York", a collection of computer made bill-boards and the spatial insight game "Kohs Block Design Test".

During the last decade Umberto is using the Dutch sprout as source for his artistically inspiration. He annually organises the art project "Do you celebrate the opening of the Brussels sprout season" in Maastricht, combining sprouts art, music, literature and commercials. Umberto is, in collaboration with colleague artists and the owner, running an art gallery in the Rechtstraat in Maastricht. His creations are sold commercially at many locations in the Netherlands. Umberto receives orders from the private sector, companies and the (local) municipality and gave his authorization for the utilization of his art in this thesis.

Samenvatting

In het afgelopen decennium heeft de productieketen voor groentes een wezenlijke gedaanteverwisseling ondergaan en is veranderd van een productiegerichte - in een klantgerichte organisatie met bijzondere aandacht voor de wensen van de consument. De huidige consument toont een groeiende belangstelling voor groentes met toegevoegde waarde en vraagt om gemakkelijk bereidbare producten die opvallen door interne kwaliteitskenmerken als smaak, gezondheidsbevorderend en voedingswaarde.

De bedrijfsketen voor groentes kan echter maar in beperkte mate inspelen op de nieuwe wensen van consumenten. Voor veel groentes ontbreken gegevens over de samenhang tussen de genoemde kwaliteitskenmerken en consumentenwaardering en hun expressie in het beschikbare rassenassortiment, vooral door een gebrek aan consumentenonderzoek, betrouwbare meetmethoden en traceerbaarheid van kwaliteitsprodukten in de handelsketen.

De leden van de bedrijfsketen voorzien momenteel gedeeltelijk in de behoeftes van de consument door "branding" van groentes met specificaties voor kwaliteitskenmerken als versheid, uniformiteit, houdbaarheid en teeltwijze maar hebben geen pasklare oplossing voor de verkoop van groentes met verbeterde smaak, gezondheidswaarde of voedingswaarde.

In dit proefschrift wordt de ontwikkeling beschreven van spruitkool met een optimale smaak voor de consument en methodes voor de ondersteuning van de productie van smakelijke spruitjes. De smaak van spruitkool wordt bepaald door de glucosinolaten sinigrine en progoitrine, zwavelhoudende inhoudsstoffen die bij een relatief laag gehalte een bittere smaak veroorzaken als intacte stof of na enzymatische afbraak. De ontwikkeling van smakelijke spruitjes is geinitieerd met de ontwikkeling van nieuwe, grootschalige meetmethodes voor sinigrine, progoitrine en de som van alle glucosinolaten in spruitjes. In hoofdstuk 2 van dit proefschrift wordt de ontwikkeling beschreven van immunochemische ELISA bepalingen met hoge specificiteit voor de glucosinolaten sinigrine en progoitrine. Beide ELISA's vormen de basis van dit proefschrift omdat hun beschikbaarheid een snelle en efficientie bepaling van

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het sinigrine en progoitrine gehalte van ouderlijnen en rassen uit het veredelingsprogramma heeft mogelijk gemaakt. Hoofdstuk 3 beschrijft een nieuwe meetmethode voor de bepaling van het glucosinolaatgehalte van spruitjes. In de bestaande referentie methode wordt het glucosinolaatgehalte van spruitjes gemeten als enzymatisch afgesplitst glucose na chromatografische verwijdering van glucose wat standaard in het monster aanwezig is en een verstorende werking heeft op de glucosinolaat bepaling. Bij onze alternatieve methode wordt tijdswinst geboekt en schaalvergroting toegepast door het storende glucose enzymatisch in plaats van met chromatografie te verwijderen, opnieuw gevolgd door de enzymatische meting van glucosinolaten. De glucosinolaatbepaling van hoofdstuk 3 heeft een belangrijke rol gespeeld in de validatie van de ELISA's voor sinigrine en progoitrine en het vaststellen van de fractie van sinigrine en progoitrine in het glucosinolaatgehalte van spruitkoollijnen en rassen.

De methodes van hoofdstuk 2 en 3 zijn gebruikt om de optimale spreiding in het gehalte van sinigrine, progoitrine en de som van glucosinolaten in het veredelingsprogramma vast te stellen en te gebruiken voor smaakonderzoek. Hoofdstuk 4 beschrijft de resultaten van diverse smaaktesten met consumenten - en expertpanels en de gevonden relaties tussen het sinigrine en progoitrine gehalte en, respectievelijk, consumentenwaardering voor en bitterheid van spruitjes. Uit ons onderzoek is gebleken dat een som van sinigrine en progoitrine van 2.2 g kg^{-1} of lager een optimale smaak garandeert voor regelmatige consumenten van spruitkool met een voorkeur voor een bepaald nivo van bitterheid. De smaaktesten waarin bitterheid van spruitjes is vastgesteld door expertpanels laten zien dat de som van sinigrine en progoitrine lager moet zijn dan 0.6 g kg^{-1} om van niet bittere spruiten te kunnen spreken. Kandidaat spruitkool consumenten, met een afkeer voor bitterheid, kunnen de omzet van spruitkool verhogen wanneer dergelijke niet bittere spruiten betrouwbaar beschikbaar komen in supermarkten en groentewinkels.

De kritische som van sinigrine en progoitrine voor optimale smaak (2.2 g kg^{-1}) en minimale bitterheid (0.6 g kg^{-1}) waren de normen voor de veredeling van verbeterde smaak in spruitkool. In

hoofdstuk 5 van het proefschrift wordt de vererving van het sinigrine en progoitrine gehalte in spruitkool beschreven. Uit onze experimenten is gebleken dat het sinigrine en progoitrine gehalte van spruitkool voor ongeveer 60% bepaald wordt door rassenkeuze (genotype) en voor 40% door omgevingsfactoren. Het sinigrine en progoitrine gehalte van spruitkoolrassen vererft intermediair aan het gehalte van de bijbehorende ouderlijnen met h^2-waarden van respectievelijk 0.72 en 1.09. De selectiedruk voor een laag gehalte sinigrine en progoitrine heeft, opmerkelijk genoeg, geleid tot een lage som van glucosinolaten en niet tot een lage fraktie van beide glucosinolaten. In een periode van 10 jaar is een assortiment F1-hybrides met een gemiddelde tot slechte smaak vervangen door goedsmakende nieuwe rassen.

De significante invloed van omgevingsfactoren op het gehalte van sinigrine en progoitine van rassen, en daarmee hun smaak, was de aanzet om deze nader te willen karakteriseren. Het is namelijk onmogelijk om spruitkool met een betrouwbare smaak te produceren bij gebrek aan controle over de omgevingsfactoren met een invloed op het glucosinolaatgehalte. In hoofdstuk 6 worden regenval, het sulfaatgehalte in de grond en de architectuur van het wortelgestel gekarakteriseerd als de parameters die het variabele sinigrine en progoitrine gehalte van spruitkoolrassen bepalen onder verschillende teeltomstandigheden. Opmerkelijk was de vondst dat de som van sinigrine en progoitrine van rassen in hoge mate gecorreleerd is aan de cumulatieve laterale wortel lengte van rassen, een parameter die waarschijnlijk gerelateerd is aan de opnamecapaciteit van sulfaat door het wortelgestel. De resultaten van hoofdstuk 6 kunnen gebruikt worden als richtlijn voor de productie van een voorspelbaar glucosinolaatgehalte in spruitkoolrassen.

Spruitkool is een voorbeeld van een kort houdbare groente als gevolg van een snel verouderingsproces na plukken. Verbetering van de houdbaarheid zal de kwaliteit van spruitkool in het handelskanaal ten goede komen doordat houdbare spruitjes hun versheid langer vasthouden en daardoor langer attractief blijven voor de consument. In hoofdstuk 7 wordt een spruitkoollijn beschreven met extreme houdbaarheid bij kamertemperatuur. De expressie van houdbaarheid is gebaseerd op de complexe

interactie tussen de auxine en cytokinine huishouding in de plant die verantwoordelijk is voor een lage basale ethyleen productie en een geremd verouderingsproces. De geniteur voor houdbaarheid is inzetbaar voor meerdere doelen en zal primair een revolutie teweeg kunnen brengen voor wat betreft de houdbaarheid van spruitkool. Bij een goede expressie van de eigenschap zal de houdbaarheid van spruitkool bij kamertemperatuur van ongeveer 5 dagen verlengd kunnen worden naar een periode van meer dan 2 weken. Tijdens het verouderingsproces van spruitjes is er sprake van afbraak van glucosinolaten en een verminderde smaak door glucosinolaat afbraakproducten als het intens bittere goitrine. De geniteur voor houdbaarheid zal de afbraak van glucosinolaten en de ontwikkeling van bitterheid in het na-oogst trajekt kunnen voorkómen.

De in dit proefschrift ontwikkelde methodes, definities voor goede smaak en bitterheid, spruitkoolrassen met goede smaak (en in de nabije toekomst goede houdbaarheid), en teeltinstructies voor de productie van goedsmakende spruitjes zijn een solide basis voor de marketing en verkoop van spruitkool met een betrouwbare smaak door leden van de bedrijfsketen van groentes. Het leggen van de basis is de verantwoordelijkheid van veredelingsbedrijven, de "branding" en verkoop van spruitkool met een goede smaak is een taak voor de andere leden van de bedrijfsketen. De "branding" van goedsmakende spruitkool is een ideale test om te zien of groentes met een betrouwbare expressie van interne kwaliteitskenmerken een toekomst hebben in de bedrijfsketen zeker gezien de verkoop van spruitkool nog maar 10 jaar geleden onder ferme druk heeft gestaan vanwege een slecht smaakimago. Een succesvolle introductie van smakelijke spruitkool op de markt zal de intrede van alternatieve kwaliteitskenmerken in andere gewassen kunnen versnellen. Bij gebrek aan succes zullen veredelingsbedrijven nog zorgvuldiger ontwikkelingen op het vlak van interne kwaliteitskenmerken afwegen op hun kans van slagen. De resultaten van dit proefschrift hebben hoe dan ook bewezen dat veredeling van gewassen met een hoogwaardige interne kwaliteit zeer wel mogelijk is.

Curriculum vitae

Hans van Doorn werd op 1 februari 1958 geboren te Geldermalsen, Gelderland, als zoon van een fruittelersgezin. Na het behalen van het VWO diploma in 1976 op de Rijksscholengemeenschap te Zaltbommel volgde een 3-jarige opleiding HBO-B biochemie bij de Hogere en Middelbare Laboratorium School in Oss. Een "verplichte" stage bij de Vakgroep Biochemie van het toenmalige B.C.P Jansen Instituut op de Plantage Muidergracht in Amsterdam bracht de liefde voor de biochemie tot volle bloei en leidde uiteindelijk tot de functie biochemisch analist bij de Interfaculteit Lichamelijke Opvoeding op het Jan Swammerdam Instituut aan de Constantijn Huijgenstraat in Amsterdam en later het Academisch Medisch Centrum (AMC) in Amsterdam-Zuid. Gedurende een periode van 10 jaar werd onderzoek verricht naar de biochemie van spiervermoeidheid en ervaring opgebouwd in de vakgebieden van Analytische Biochemie en Inspanningsfysiologie. In 1984 werd in de avonduren en het weekend begonnen met de studie Scheikunde aan de Universiteit van Amsterdam. Deze studie werd in zomer 1988 afgerond met een specialisatie in de biochemie. In hetzelfde jaar werd de Universiteit verruild voor een functie bij de sectie Biochemie van het veredelingsbedrijf Zaadunie BV, het huidige Novartis Seeds BV, in Enkhuizen. Sinds 1988 werkt hij aan kwaliteitskenmerken van groentegewassen die de smaak, textuur, houdbaarheid en teeltprestaties bepalen en ondersteunt de veredeling en marketing van kwaliteitsbepalende eigenschappen in het gewassenassortiment van Novartis Seeds BV.

Nawoord

Het nawoord is een ideale gelegenheid om de mensen die een tastbare bijdrage hebben geleverd aan mijn proefschrift te bedanken. Ik wil in de eerste plaats Annelies, mijn vrouw en vriend, bedanken voor haar trouwe steun tijdens het schrijven van dit proefschrift. Jouw zorg, toewijding en geduld gaven mij de ruimte om dit werkstuk te voltooien. In dit proefschrift wordt onderzoek beschreven wat is uitgevoerd over een periode van 10 jaar en is ondersteund door collega's die al weer andere wegen bewandelen binnen of buiten de wetenschap. Jille, Natasja en Marianne, bedankt voor jullie bijdrage aan het geheel.

Dit proefschrift is in zekere zin een co-productie van Novartis Seeds en de Universiteit van Wageningen. Op deze manier wil ik mijn waardering uitspreken voor de enthousiaste en kritische bijdrage van Wim Jongen en Linus van der Plas aan de vervaardiging van dit proefschrift. Het lijkt me moeilijk om de kwaliteit van onderzoek te toetsen wat in eerste instantie niet door jezelf geinitieerd is. Ik hoop dat we na mijn promotie kunnen blijven samenwerken in projecten die onze gezamelijke belangstelling hebben.

Dit proefschrift is mijn hobby maar een met een serieuze ondertoon. Onze vondsten hebben gedurende lange tijd de status van "nog even geduld" gehad maar konden wegens succes uiteindelijk de wetenschap gaan dienen in de vorm van publicaties en dit proefschrift. Dit proefschrift zou niet tot stand gekomen zijn zonder de gedreven inbreng van het veredelingsteam van spruitkool: Jan de Nijs, Ko Broer en Fred Oud. Jongens, bedankt voor jullie waardevolle inbreng, discussies en vooral jullie selectiewerk wat geleid heeft tot de huidige generatie spruitkoolrassen en de vrijheid om te kunnen publiceren.

Het proefschrift zou ook niet tot stand zijn gekomen als ik indertijd niet daadkrachtig door Ad Kool aangenomen zou zijn in mijn huidige functie. Ik wil het toenmalige en huidige management van Novartis Seeds bedanken voor het verlenen van de middelen en mogelijkheden om dit proefschrift te kunnen realiseren. De inhoud van dit proefschrift is in belangrijke mate beinvloed door mijn voormalige programmaleider Gerrit-Jan van

Holst, mede-auteur van vele publicaties, advokaat van de duivel, maar vooral een bron van inspiratie in tijden van nood. Gerrit-Jan bedankt voor je sturende hand en je waardevolle discussies en suggesties. De meeste metingen en experimenten in dit proefschrift zijn zonder twijfel uitgevoerd door Gert van der Kruk, mijn steun en toeverlaat in de wereld van biochemie en analyses en de laatste der Mohikanen in onze onderzoeksgroep. Gert, zonder jou zou het proefschrift veel dunner en minder zijn geweest.

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