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Role of physiological factors in tulip bulb scale micropropagation

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Stellingen

1. Het optreden van oxidatieve stress is *niet* altijd negatief voor weefsels maar kan daarentegen zelfs de regeneratie bevorderen.

(R.H. Burdon, *Biochem. Soc. Trans.* **24** (1996):1028-1032; Y.J. Suzuki, H.J. Forman, A. Sevanian, *Free Rad. Biol. Med.* **22** (1997): 269-273)

2. De opheldering van regeneratie specifieke processen is nodig voordat knelpunten in het verkrijgen van goede regeneratie kunnen worden opgelost.

3. Het verschil tussen de uitkomsten van biotoetsen en HPLC-metingen bij de bepaling van het tulipalinegehalte geeft aan dat precieze kennis over reactieomstandigheden en reactiviteit noodzakelijk is voor de interpretatie van de resultaten van biotoetsen.

(Dit proefschrift; J.C.M. Beijersbergen, proefschrift Leiden, 1969).

4. Het verschil in resultaat bij het gebruik van verschillende organen of bij identieke organen met een verschillende leeftijd suggereert dat bij de regeneratie van planten de differentiatiegraad een van de belangrijkste factoren in het regeneratieproces is.

5. Het feit dat president Clinton in zijn State of the Union van 1995 onderzoek naar "stress bij planten" als voorbeeld noemde van onderzoek dat onzinnig is en dus geschrapt dient te worden is een indicatie van de onbekendheid van de buitenwereld met de thema's en termen uit het botanisch onderzoek.

6. Ondanks hun voedingswaarde en het veelvuldig eten van tulpen in de winter van 1944-1945, zijn waarschijnlijk emotionele overwegingen de oorzaak van het feit dat tulpen geen vaste plaats in ons voedingspakket hebben verkregen.

7. De beschrijving van de schepping van Eva uit een rib van Adam is waarschijnlijk de vroegste literatuur over klonering; tevens is hieruit duidelijk dat somaclonale variatie ook toen al een rol speelde.

8. Iets ontdekken bestaat uit zien wat anderen zien en bedenken wat een ander niet heeft bedacht. (Albert Szent-Györgyi, Nobelprijswinnaar 1937). Wetenschap is het interpreteren van het bedachte en vooral het overtuigen van anderen van de interpretatie.

9. Het grootste nadeel van een optimistische houding is de verhoogde kans op tegenvallers.

10. Efficiency is de meest geprezen vorm van luiheid.

11. De watersnood van 1995 had aanleiding kunnen zijn tot het gezegde "als het kalf geëvacueerd wordt, verhoogt men de dijken".

12. Openbaar vervoer zal nooit een aanvaardbaar alternatief voor autogebruik worden in gebieden waar rekening moet worden gehouden met geografische barrieres zoals rivieren: deze situatie verandert niet door het verbeteren van de infrastructuur.

13. Ook pc's zijn gevoelig voor stress: ze vertonen de meeste storingen als ze het hardst nodig zijn.

Stellingen behorende bij het proefschrift "Role of physiological factors in tulip bulb scale micropropagation".

Wageningen, 2 december 1997. Maarten W.P.C. van Rossum

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List of Abbreviations

cv.	cultivar
DW	Dry Weight
FW	Fresh Weight
LOX	Lipoxygenase
MDA	Malondialdehyde
PAL	Phenylalanine Ammonia Lyase
PPO	Polyphenoloxidase
PUFA	Poly Unsaturated Fatty Acid
SOD	Superoxide dismutase
TBArs	Thiobarbituric acid reactive substances

Voorwoord

Toen ik in 1992 begon met mijn promotieonderzoek aan de vakgroep Plantenfysiologie viel dit net samen met de langverwachte verhuizing naar de nieuwbouw van de vakgroep ('de Banaan') gevolgd door de nog langer verwachte fusie met de voormalige vakgroep Plantenfysiologisch Onderzoek. Dit was het begin van een hectische, maar zeer plezierige tijd bij de "nieuwe" vakgroep Plantenfysiologie. Dit eerste jaar kan gezien worden als een tropenjaar, de combinatie van de hectiek van een nieuw onderzoek, gecombineerd met de niet altijd ideale temperatuursbeheersing tijdens warme zomers in de banaan hebben hiervoor zorg gedragen. Een extra complicatie hierbij was dat de meeste experimenten ingezet zijn tijdens de zomermaanden, resulterende in het zwetend verrichten van mijn proeven. Als gevolg hiervan zal ik nooit meer tegen een tulp aankijken als voor dit onderzoek. In deze plezierige werkomgeving heb ik mijn project -wat niet altijd even voorspoedig liep- af kunnen ronden. Dankzij de goede sfeer en collegialiteit binnen de vakgroep was het altijd mogelijk om op de moeilijke momenten de juiste richting en motivatie weer terug te kunnen vinden. Dit was mede terug te vinden in de lunch pauzes waarbij onder het genot van een heerlijk kopje koffie in onze koffiehoek waarbij gezeten op het illegaal binnengebrachte (antieke?) bankstel een veelvoud van onderwerpen de revue passeerden.

In mijn periode op de vakgroep heb ik prettig kunnen samenwerken met een groot aantal collega's en studenten die ik niet allen afzonderlijk hier wil noemen. Wel wil ik een extra woord van dank aan mijn promotor, Linus van der Plas richten die me vaak weer op het juiste pad heeft gezet op momenten dat ik het allemaal even niet meer zo duidelijk zag en hierdoor dus onschatbare steun heeft gegeven. In de periode waarin dit onderzoek is verricht hebben een aantal studenten meegewerkt aan verschillende aspecten van dit onderzoek. Hoewel hun werk niet altijd even duidelijk is terug te vinden in dit proefschrift heeft hun werk toch bijgedragen aan de gedachtenvorming en de bepaling van de richting van dit onderzoek. Daarom wil ik hier Joyce Kerstens, Mark Alberda en Wieger Wamelink van harte bedanken voor hun bijdrage aan het onderzoek. Speciaal wil ik Joyce nog bedanken voor haar medewerking bij het inzetten van de laatste grote proef in Lisse waarbij een aantal

dagen met veel uren werden gemaakt in de flow-kasten maar ook bij het bereiden van de vele liters media en de honderden petrischalen. Verder wil ik Kim Frederiks nog bedanken voor het nalopen van dit proefschrift op taal en grammaticafouten.

Ook in Lisse hebben enkele mensen bijgedragen aan dit proefschrift door kritische opmerkingen te plaatsen tijdens onze regelmatige besprekingen van de resultaten en de planning van de verdere experimenten. Daarom wil ik Merel Langens-Gerrits, Henk Gude, Piet Boonekamp en Jacques Beijersbergen van het LBO en Geert-Jan de Klerk van het COWT bij deze bedanken voor hun waardevolle bijdragen.

Chapter 1

General introduction

Tulip

A plant which strongly determines the image of the Netherlands is the tulip. Although not a native plant, it is a crop which has been grown in this country for more than 400 years and has become economically very important. The first tulip to flower in the Netherlands was grown by the herbalist Clusius in the botanical garden of Leiden in 1594 but the tulip was originally brought here from the middle east (Turkey, Afghanistan) where it can be found in mountaineous areas. Tulips became a very popular flower and it did not take long before a flowering trade, accompanied by selective breeding was established. In the seventeenth century this resulted in the so-called "tulipomania" when tulips were sold at extremely high prices. From this time on tulip has been an important part of Dutch horticulture products and numerous cultivars have been developed and are being sold throughout the world. The Netherlands is among the world leaders in total tulip production both for flowers and bulbs (LeNard and De Hertogh 1993).

Tulip is a monocotyledonous plant and a member of the Liliaceae family. The genus Tulipa comprises about 125 species divided over two sub-genera, Eriostemones and Leiostemones. Tulipa gesneriana L. and most of present day tulips of commercial interest belong to the subgenus Leiostemones although the origin of several hybrids developed over time is not completely clear (LeNard and De Hertogh 1993).

The production of tulip flowers and bulbs was only possible with the application of large amounts of pesticides and soil desinfectants since all bulbous crops, like tulip, are quite sensitive to diseases, so tulip culturing was among the most polluting sectors in Dutch agriculture and therefore obliged to develop less polluting methods. In the end of the 1980's a plan was devised to reduce the use of chemicals with 70-80% by the year 2000. By using alternative culturing methods a 50% reduction has already been achieved. For further reduction, tulip growers need new varieties with increased resistance against pests. Next to this environmentally inspired need for new varieties, also the search for new cultivars (e.g. new flower colors and shapes) goes on, to maintain the present position of Dutch tulips on the world market. Problems encountered in this respect are:

- the relatively slow propagation rate of tulip via traditional methods.
- the slow growth rate of the tulip.

- the lack of knowledge on the infection process for a large number of pathogens.
- the limited knowledge on the existing resistance to diseases in the various cultivars.
- the difficulties encountered in transformation of bulbous crops.

Life cycle of tulip

For the production of new varieties of tulip by classical breeding methods, the new flower must be produced starting from seed after crossing the plant: the embryo produces a plant with a cotyledonary leaf, a primary root and a so-called "dropper" (principally a hollow diverticulum). This dropper grows down into the soil and eventually produces a small bulblet. This bulblet needs another four to five growth seasons before the bulb is big enough to produce a flower; usually the evaluation of the new tulip takes place at this stage. If the new variety is satisfactory it has to be propagated further in order to create a stock for culturing and forcing. This propagation is done vegetatively: between the different bulb scales, vegetative buds develop that can give rise to new bulbs. The propagation rate is dependent upon the number of scales. The rate of propagation which can be obtained via this method is very slow: normally about 4-5 new bulbs per mother bulbs are produced annually (LeNard and De Hertogh 1993). With this propagation rate it will take about 25-30 years before a new variety can be introduced upon the market in a commercially successful way. The length of this period can cause severe problems for the introduction of the desired new varieties. Because of the importance of tulip bulb cultivation, alternative ways to obtain and propagate tulips are being developed. Micropropagation of tulip, using tissue culture techniques might be such an alternative that will shorten the period to 7-10 years before new cultivars can be introduced (De Vroomen 1995).

Plant Tissue Culture

Since all plant cells are theoretically able to regenerate new plants (concept of "totipotency" of plant cells), micropropagation via tissue culture might be a potent tool to produce large numbers of (genetically identical) plants starting from only a small amount of material. In horticulture the use of micropropagated material has got a wide range of applications. Advantages are the uniformity of the material, the possibility to produce virus-free plants and the reduction of the need for large

nurseries. Disadvantages are the need for specialized tissue culture facilities, the amount of labour needed in the process, the possibility of somaclonal variation and the recalcitrance of some species or tissues. Tissue culture on a commercial scale is used for the propagation of a large range of a.o. vegetables, ornamental plants, pot plants and even woody species. In 1995 53.8 million plants were produced via tissue culture in the Netherlands and a further 77.3 million plants were imported (Pierik 1996). On a non-commercial scale, tissue culture is applied for the propagation of rare species for conservation purposes. Although major advances have been made, a number of crops remain recalcitrant. In general, to improve or develop tissue culture protocols for recalcitrant species 'trial and error' experiments are the normal strategy. Methods successful for other crops are tested; these may comprise variations in starting material (different organs, different ages, different pre-treatments), hormone or medium salt composition, light conditions, temperature variations, medium changes and additions of a wide variation of substances reported to act beneficiary in other systems (Kuroda et al. 1991; Flores et al. 1994).

Micropropagation of tulip

Tulip has turned out to be a crop which is very hard to propagate in vitro. Some successful attempts have been reported using different starting materials (LeNard 1989; Nishiuchi 1986). Most attention however has focused on the use of bulb scale explants and tulip stalk explants; the latter appeared to be the most successful starting material until now. The use of stalk material was recently subject of a number of studies: a histological study of the regeneration process has been published by Chanteloube et al. (1993), other groups have succeeded in the improvement of the performance of this starting material (Kuijpers and Langens-Gerrits 1997). Stalk explants have also been successfully used for transformation of tulip material (Wilmink 1996). Nevertheless this tissue still presents us with problems, e.g. the use of stalk material is hampered by the limited time of the year that it is available and the limited amount of material that can be used for initiation of adventitious shoots. A number of these problems have been solved by using "ice-tulips" (tulips stored at -2°C, see Gude and Dijkema 1997) which are available throughout a much longer period. The total number of tulip plants propagated in vitro (11240 in 1995) is therefore very small when compared to the total amount of

micropropagated plants and even to the amount of micropropagated bulbous plants either produced or imported in the Netherlands (6 and 33.6 million respectively, Pierik 1996).

The use of bulb scale material as a basis for micropropagation of tulip has proven to be even more cumbersome. However, this material would be attractive as starting material as it is principally available almost throughout the year. The number of explants which can be cut from the bulb scales is high compared to stalks. Previous research (Koster 1993) did show, however, that without special treatments satisfactory regeneration of tulip bulb scale explants is only possible within a very limited period of the year. Outside this period the bulb scale explants suffer from severe browning and show a very poor viability.

Free radicals in plant tissue culture

Since loss of viability and the browning of plant tissue culture systems are often related to free radical damage (Benson et al. 1992; Creemers-Molenaar et al. 1992), we investigated the role of oxidative stress caused by free radicals during micropropagation starting with tulip bulb scale explants. A number of reactive oxygen species are known to occur in plant cells during tissue culture (Housti et al. 1992; Ishii 1988; Piqueras et al. 1996). They are produced by interruption of the reaction chains in metabolic pathways (e.g. in mitochondrial, chloroplastic or other membrane-linked electron transport chains) (Alscher et al. 1997) and might lead to a number of chain reactions. In these chain reactions a number of different oxygen radicals can be produced. The first radical usually is the superoxide radical ($^{\bullet}O_2^-$), followed by a number of subsequent reactions in which other reactive species are produced. The best known reaction is the so-called Fenton reaction: in the reaction of $^{\bullet}O_2^-$ with Fe^{3+}, Fe^{2+} and O_2 are produced. This Fe^{2+} reacts with hydrogenperoxide and as a result the very reactive $^{\bullet}OH$ (hydroxyl radical), OH^- and O_2 are formed. The hydroxyl radical is one of the most reactive radicals known and can initiate a number of reactions with all types of organic compounds present in the cell. These reactions lead to the biological inactivation of these compounds. Among the reacting substances are enzymes which get cross-linked, while also DNA and membranes can get damaged. The effect of free radicals on membranes has been well documented and is subject to a number of studies (Gardner 1989; Van Bilsen et al. 1994). It is related to the chemical nature of

the membranes which contain a relatively large amount of polyunsaturated fatty acids (PUFAs) which are the main target of free radicals. By reaction of the free radical with the PUFAs the fatty acid gets damaged and forms a PUFA-radical (initiation reaction) which attacks other PUFAs (in the presence of oxygen) resulting in a chain reaction. The process is stopped if two free radicals react with each other and a stable compound is formed (termination reaction). If the process of lipid peroxidation is not terminated in time, the membranes of the cell can suffer serious damage. The resulting leaky cell membranes may cause loss of electrolytes and even proteins. Finally no proper biological functioning of the cell can be expected any more. A number of possible reactions and products occurring during free radical damage of membranes is shown in figure 1.1. Free radical processes in plant tissues have been thoroughly summarised by Halliwell and Gutteridge (1985) and Benson (1990). They are described to occur during infection with pathogens, flooding, heat stress, wounding, chilling injury and senescense. These processes are often accompanied by lipoxygenase activation and by the production of MDA, a lipid breakdown product which is often used as a marker for oxidative damage (Benson 1990; Leshem 1988).

The combination of all the damaging processes initiated by free radicals, results in browning of the tissue. If this occurs in tissue culture the result often is a lack of regeneration due to irreversible loss of vitality and death of the tissue.

Oxygen stress defence mechanisms in plant cells

Because of the wide range of processes which can induce oxygen stress, aerobic organisms have developed a range of defence mechanisms for coping with this stress. The defence mechanisms can be divided into two different types: enzymatic and non-enzymatic. The non-enzymatic defence mechanisms are based on the production of a number of anti-oxidative substances which are able to quench free radical species. The best known anti-oxidant in biology and medicine is α-tocopherol (vitamine E). Its biological function is reviewed by Fryer (1992). Another well-known biological antioxidant is glutathion. This tripeptide is present in the cell in an oxidized and a reduced form and is cycled via enzymatic systems (de Vos et al. 1994). Other antioxidative species present in the cell comprise e.g. vitamin C and quercetin. The enzymatic defence system is formed by a number of different enzymes, sometimes performing complementary tasks in the removal of reactive oxygen species or

products. The first enzyme in the cycle is SOD; this enzyme reacts with $^{\bullet}O_2^-$ under the formation of hydrogenperoxide. For the processing of hydrogen peroxide, catalase and a number of peroxidases can be used (figure 1.1).

Since oxidative stress plays such an important role in the determination of the vitality of plant tissues, tissue culture performance can often be enhanced by the addition of antioxidants to the nutrient medium. A wide range of additions have been tested (Creemers-Molenaar et al. 1992; Flores et al. 1994). Their working mechanisms

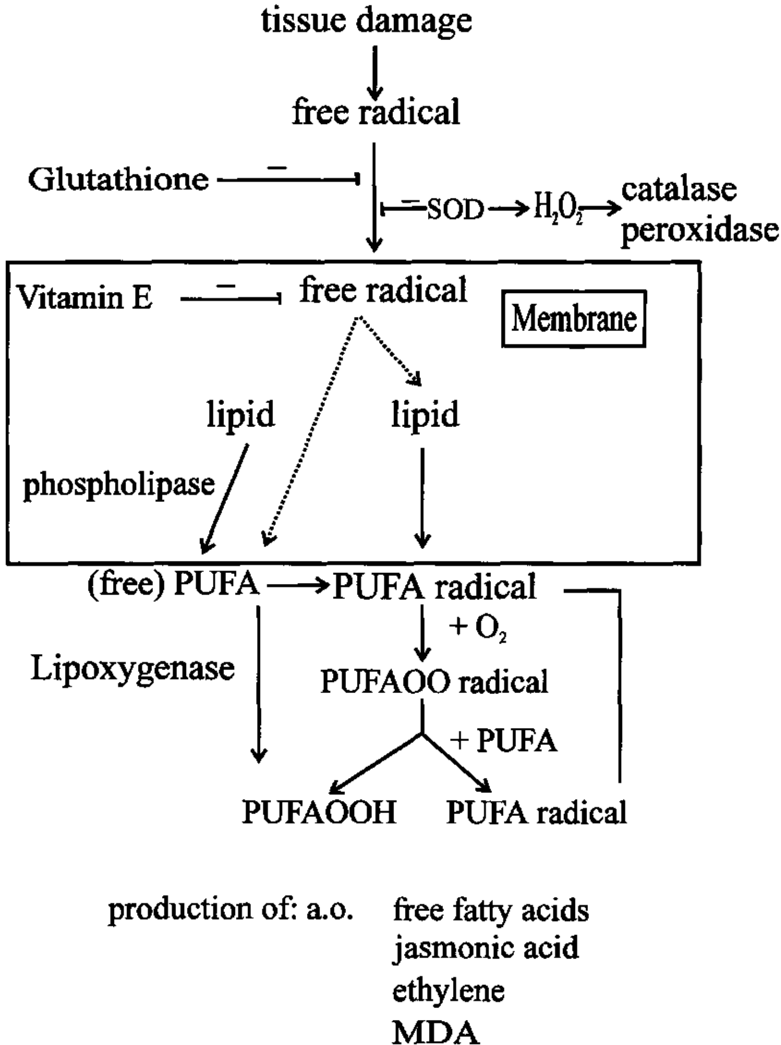

Figure 1.1 Schematic overview of some reactions involved in oxygen stress-related processes and the mode of action of a number of protective mechanisms. A number of these processes will occur at the boundary of membrane and cytoplasm.

are not always clear since a lot of these additions have been found by trial and error methods. They might counteract directly oxidative species (e.g. activated charcoal), or absorb reaction products like phenolic substances (e.g. PVP, polyvinylpyrolidone).

Because of the important role of oxidative damage and the wide body of literature in which tissue culture performance could be improved by antioxidants, we evaluated the role of oxidative damage in our tissue culture system: the bulb scale micropropagation of tulip. We hypothesized that tulip bulb scale explants are extremely sensitive to oxidative damage, leading to loss of vitality and finally death of the explant. To test this hypothesis we evaluated the physiological response of different tissues or during modified experimental conditions like the application of antioxidants or changed oxygen levels.

Tulipaline in tulip tissues

Plant tissues often contain secondary metabolites which perform a function in the protection of the cells to unfavourable environmental conditions. They may function as repellent to animals which feed upon the plants or play a role in the resistance to infections by fungi or bacteria. In tulip a reasonably well characterised secondary metabolite is tulipaline A (α-methylene-γ-butyrolactone) although other tulipalines can also be found (Beijersbergen 1969; Tschesche et al. 1968). Tulipaline is not found exclusively in tulip but also in other Liliaceae (Slob 1973; Slob et al. 1975). This unsaturated lactone is reported to have a role in the resistance against the tulip pathogen *Fusarium oxysporium f. tulipae*. The lactone is described to be very reactive and therefore toxic to the fungus but probably also to the tulip tissue itself. Therefore, it has been suggested that tulipaline is present in the plant as a non-toxic glycoside. In the case of tulipaline A this is a glucoside of α-methylene-γ-hydroxybutyric acid called tuliposide A (Tschesche et al. 1968; Christensen 1995a,b). Tuliposide A is thought to be produced out of a special amino acid, 4-methylene glutamine (Beijersbergen 1969), an important transport amino acid in the tulip (Lambrechts et al. 1992). Tuliposide A is present in tulip bulb scales (Tschesche et al. 1969) but also in other tissue types (e.g high concentrations are found in pistils). Penetration of a fungus in bulb scale tissue leads to the release of tulipaline A (possibly from its precursor tuliposide A) by release and subsequent lactonisation of α-methylene-γ-hydroxybutyric acid which results in the inhibition of the infection by

the formation of a layer of dead cells at this penetration site (Beijersbergen and Lemmers 1972).

It has been postulated (Beijersbergen 1969) that wounding of bulb tissue (fungal penetration or cutting of explants) results in decompartimentalisation which leads to (enzymatic) liberation of tulipaline A from the tuliposide. It is assumed that tulipaline A is too reactive to be present in biological systems without causing severe problems in the cells, meaning that it is present in the precursor form and is separated from the tuliposide degrading enzyme by localisation in different compartments in the cell.

The reactivity of tulipaline A is confirmed by results from dermatological research which has identified tulipaline A as a major factor in several contact dermatitis cases which occur on people working in the flower industry (Gette and James 1990; Hausen et al. 1983; Hjorth and Wilkinson 1968). Also in these cases the reactivity of tulipaline is thought to cause the contact allergy ("tulip fingers") for the people working with this crop.

Therefore our second working hypothesis was that during cutting and incubation of bulb scale explants a release of tulipaline A from tuliposide A might take place. Exposure of the tissue to the reactive compound will then lead to severe browning and decrease of the viability of the tissue. As a result of the decreased viability no regeneration of new shoots on bulb scale explants is to be expected.

Scope of this thesis

In this thesis we attempt to elucidate the physiological factors which cause the unsatisfactory regeneration of tulips via micropropagation starting from bulb scale explants. In contrary to standard tissue culture practice we did not only attempt to improve tissue culture performance of tulip bulb scale explants by trial and error methods (by differing incubation conditions) but additionally measured the physiological responses of the tissue and by doing so studied the influence of the changed conditions on the physiological processes thought to be involved in the vitality of plant tissue during incubation *in vitro*. In this way we tried to evaluate the key problems which play a role in the poor viability of tulip bulb scale explants.

Two hypotheses were tested during the research described in this thesis. In the first hypothesis we focused on the role of oxidative stress. Oxygen stress, especially during the process of cutting the explants, might evoke a chain of reactions eventually leading to vitality loss and death of the explants. The possibility that tulip bulb scale tissue might be extremely sensitive to such oxidative damage was tested. Therefore, the physiological response of regenerating and non-regenerating bulb scale explants was investigated and compared with other regenerating systems such as tulip stalk explants and lily bulb scale explants. A survey was made of the changes in physiological parameters (e.g. activities of enzymes involved in oxygen stress reactions) in regenerating or non-regenerating tulip tissue under standard incubation conditions (chapter 2). Subsequently, the growth conditions were changed by incubation of various tissue culture systems in high and low oxygen and following the response of the explants; in this experimental setup, an attempt was also made to determine in which phase of the incubation oxidative damage might play a role (chapter 3). The physiological response of bulb scale explants was also investigated in the presence of various specific additives thought to interfere with the oxygen stress response to the nutrient medium (chapter 4).

In the second hypothesis we studied the possible role of tulipaline. Since cutting the explant, results in the creation of a relatively large wound surface, it might result in the release and/or production of this reactive lactone which can be damaging to the tissue (chapter 5).

In the general discussion (chapter 6) both hypotheses are evaluated and their possible role in explaining the physiological basis for the problems in tulip bulb scale micropropagation is discussed.

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Chapter 2

Role of oxidative damage in tulip bulb scale micropropagation

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Summary:

The activation of oxygen stress-related enzymes was compared in regenerating and non-regenerating tulip bulb scale explants and regenerating stalk explants. The phospholipid composition of scale explants showed an increase of linolenic acid (from 1 to 15%) and a decrease in linoleic acid (from 70 to 55%); after incubation it was comparable to that of stalk explants in which no changes were observed. In all tested systems an increase in activity of catalase, peroxidase, SOD, lipoxygenase, polyphenoloxidase and phenylalanine ammonia lyase, was observed during incubation of the explants. The reaction could be divided into two phases. The first one (observed for scale explant lipoxygenase and to a lesser extent for SOD) occurred rapidly (1-2 hours) after cutting the explants and seemed wounding related. In the second phase (observed for all enzymes), starting during the first week of incubation, wound healing and regeneration could be observed. The activation of catalase, peroxidase and phenylalanine ammonialyase was comparable in all tested systems and seemed not to be related with the differences in tissue culture performance. In the second phase the activities of lipoxygenase, peroxidase, catalase and phenylalanine ammonia lyase decreased in regenerating explants while in non-regenerating explants they remained high.

Our conclusion from these results is that oxidative damage is not the prime cause of the low regenerability of tulip bulb scale explants.

Introduction

Tulip bulbs are generally propagated vegetatively and have a natural propagation factor of about 4-5 new bulbs per bulb. Therefore, it takes 25-30 years before new varieties differing in colour, size, shape or in resistance to diseases can be successfully introduced into the market. Micropropagation *via* tissue culture might solve the problem of the slow propagation rate. Principally in tissue culture every type of tissue can be used as a starting material, but for *in vitro* culture of tulip generally two types of tissue are of interest: bulb scales and stalks. Successful micropropagation of tulip from stalk tissue has been shown by a number of groups (Taeb and Alderson

1990; Le Nard 1989; Rice et al. 1983). Micropropagation of tulip starting from bulb scale explants has been proven possible (Nishiuchi 1986; Koster 1993), but the use of bulb scales still presents a number of problems. The period in which successful regeneration can be obtained from bulb scale explants is still very limited (July-August). Especially outside this period the explants show very poor viability, resulting in severe browning and eventually death of the explants; this might be the cause of the often complete lack of regeneration during this period. The physiological basis of this problem is one of the central themes of our research.

In this paper, we focus on some aspects of the stress-related wound response of the explants. The cutting of a bulb scale explant results in the creation of a relatively large wound surface and therefore in a massive wounding reaction, involving a possible oxidative stress-response (Halliwell and Gutteridge 1985). Furthermore, in a closely related crop like lily a relation between oxidative stress (ethane and ethylene production) and regeneration has been suggested (van Aartrijk et al. 1985). In this process free radicals are produced and peroxidative damage can occur, leading to activation of peroxidases, catalase and SOD (Leshem 1988; Scandalios 1993; Bartoli et al. 1995; Olmos et al. 1994). SOD has a function in the removal of activated oxygen species, simultaneously producing hydrogen peroxide as a reaction product. This hydrogen peroxide is removed by the activity of catalase and peroxidase. Peroxidase has also a function in the formation of cell wall components (Halliwell and Gutteridge 1985). Also phenolic substances are formed which are oxidised by polyphenoloxidase, resulting in polyphenolics which cause the visible browning of the tissue. Phenolics are known to exhibit toxic effects to cells in tissue culture (Glass and Dunlop 1974; Bhat and Chandell 1991; Housti et al. 1992). Phenylalanine ammonia lyase (PAL), an enzyme which is generally induced during the stress response of a tissue, plays a role in the formation of these phenolics (Davies 1972; Hyodo et al. 1978). Other enzymes that are induced upon wounding, comprise lipoxygenase that causes oxygenation of unsaturated fatty acids possibly leading to a subsequent increase in the degree of saturation of membrane lipids (Gardner 1989). This might result in a lower fluidity of the cell membranes which get more leaky. Such leakage of e.g. ions or proteins might contribute to the death of the cells and the explant. As a result of lipoxygenase activity a number of substances is produced like e.g. malondialdehyde (MDA), jasmonic acid, ethylene and a number of other volatile substances.

We hypothesized that the cause of the poor regeneration of tulip bulb scale explants might be an insufficiently functioning mechanism for dealing with oxidative stress, leading to a too low activation of SOD, peroxidases and catalases and an increased damage to membranes as e.g. shown by production of TBArs and phenolics. Therefore, we monitored a number of physiological reactions which are correlated with these stress responses, such as changes in membrane composition and activities of relevant enzymes. A comparison was made between bulb scale explants which showed regeneration and/or callus formation, and explants with poor viability showing severe browning and no signs of extensive cell division. For comparison, stalk explants showing good regeneration and callus formation were used.

Material and methods

Tulip material

Tulip bulbs (*Tulipa gesneriana* L.) cv. Apeldoorn were field grown and harvested in early July. After drying they were stored at 30°C for two to four weeks. Immediately after this period explants were cut (end of July / August). For use in the stalk explant experiments, bulbs were stored at 20 °C until the middle of October followed by 17 °C until November. The bulbs were planted in pots with soil and stored at 9 °C for 6 weeks, where rooting and some sprout development occurred. After this treatment the pots were stored at -2 °C prior to use (Gude and Dijkema 1997).

Tissue culture

Tulip bulb scale explants

After removal of the brown skin, bulbs were longitudinally cut in four parts. After rinsing first with 70% ethanol and then with water to remove remaining ethanol (each 30 seconds), the bulb parts were sterilised in 1% hypochlorite solution for 30 minutes. After sterilising, the bulbs were rinsed three times in sterile water. The second and third outermost scales were used as starting material. The explants were cut from the basal part of the scale, just above the basal plate; in our experiments we used only the lower two explants from each scale, so 16 explants per bulb were cut. Explants were ± 2.5 mm thick and weighed 70-100 mg. Explants were put on the

medium with the basal side down and grown on full-strength MS medium (Murashige and Skoog 1962), containing 3% sucrose, 0.5 g/l casein hydrolysate (Gibco), 0.1 g/l myo-inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 1 mg/l 2,4-D, 1.5 mg/l BAP and 6 g/l agar (BBL) (Gude and Dijkema 1997); the pH was adjusted to 6.0 prior to autoclaving the medium. The explants were cultured for 10 weeks at 20°C in the dark.

Tulip stalk explants

The stalks were isolated from the bulbs at the end of December, the top was removed and the stalk was surface sterilised as described for the bulb scale explants. Six explants per stalk were cut around the first node, thickness ± 1-1.5 mm. The explants were incubated for 10 weeks on MS medium as described for bulb scale explants (basal side down).

Incubation and evaluation of explants

From every bulb or stalk the explants were divided between two petridishes. From these two sets of explants, one set was sampled, weighed and stored at -80°C in the course of the incubation, while the other set remained in tissue culture for evaluation of the regeneration. In this way, the "vitality" of individual bulbs could be determined.

After 10 weeks all explants were evaluated and scored for callus or shoot formation and for browning. The reaction of the explants was scored, according to the following classifications: normal callus formation (1 point), extensive callus formation (2 points), begin of shoot formation (2 points) or shoot formation (4 points). For browning a negative score was assigned to the explant (-1 point). Each explant was individually scored, and the mean score for the explants derived from one bulb was calculated. As from each bulb the performance of the explants was individually scored and samples of these explants were taken throughout the course of incubation and individually stored at -80°C , it is possible to determine the changes in enzymatic activities separately of "growing" (high scoring) and "non-growing" (low scoring) explants.

Sample preparation

Samples, consisting of 6 explants per bulb were taken at different time intervals during the tissue culture period, frozen in liquid nitrogen and subsequently stored at -80 °C. At each sample point explants from ten bulbs were individually sampled and frozen until analysis.

For each data point material from three bulbs was pooled and ground in liquid nitrogen. From this material two samples of 500 mg were taken and transferred to 1 ml 0.05 M potassium phosphate buffer pH 7.0, containing 1 mM $CaCl_2$, 1 mM KCl and 1 mM EDTA (Benson et al. 1992a). The resulting extracts were used for enzyme activity determinations. Enzyme activity of each extract was determined in two replicate experiments.

Enzyme activity determinations

Catalase activity was determined by incubation of 20 µl of the enzyme extract in 1 ml of 0.1 M potassium phosphate buffer pH 7.0 containing 27 mM of hydrogen peroxide. The disappearance of hydrogen peroxide was spectrophotometrically followed at 240 nm in a thermostated spectrophotometer (Shimadzu UV1601PC) at 30°C (Benson et al. 1992a).

Peroxidase activity was determined spectrophotometrically by measuring the rate of increase in oxidised guaiacol at 470 nm in a thermostated spectrophotometer at 30 °C, using 15 mM guaiacol in 0.1 M phosphate buffer, pH 5.0, and 27 mM hydrogen peroxide (Benson et al. 1992a).

Lipoxygenase activity was determined by measuring the formation of conjugated dienes at 234 nm using 10 mM linoleic acid as the substrate in a thermostated spectrophotometer at 30 °C. 1 Unit of LOX activity is defined as the amount of enzyme forming 1 µmol of hydroperoxidiene per min. (Sanz et al. 1992).

Superoxide dismutase activity was determined by a modified method according to Beyer and Fridovich (1987). With this method, the inhibition of reduction of NBT (nitro blue tetrazolium) by enzyme extracts is measured. A dilution series of the enzyme extract (2, 4 and 8 times diluted) was pipetted into a microtiter plate. The plate also contained wells without extract and with either 1 or 10 units commercially available SOD (Sigma). One unit of enzyme activity is defined as the amount of enzyme capable of 50 % reduction of cytochrome c in a coupled system

with xanthine and xanthine oxidase; in our system this is identical to a 50 % inhibition of total SOD-inhibitable NBT-reduction. To all wells, 0.2 ml of a solution containing 0.05 M potassium phosphate buffer pH 7.8, 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA and 0.025% triton X100 was added. To start the reactions 20 μl of 10 mM riboflavin was added and at the same time the plate was placed under fluorescent light for 1.5 minutes. After this period, the microtiter plate was transferred to the dark, riboflavin was added to the wells containing the blank, and absorbance was determined at 630 nm. SOD activity was determined by calculation of the amount of extract, which gave 50 % inhibition of NBT-reduction.

Polyphenoloxidase activity was determined by measuring the oxidation of 10 mM L-DOPA in 50 mM potassium phosphate buffer pH 9.0, the oxidized product showing absorption at 470 nm. The polyphenoloxidase activity was measured during the first two minutes in a thermostated spectrophotometer at 30 °C (Söderhall 1995).

Phenylalanine ammonia lyase activity was determined by a modified method according to Zucker (1965) by incubating 50 μl of extract in 0.1 M borate buffer, pH 8.8, containing 60 mM phenylalanine and following the increase in absorbance as a result of the formation of cinnamic acid at 290 nm in a thermostated spectrophotometer at 40 °C. 1 Unit of PAL activity is defined as the amount of enzyme causing an increase of absorbance of 0.01 (Zucker 1965).

All enzymes activities are presented on a fresh weight basis (per gram of FW of starting material).

Protein determinations

Protein content of the samples was determined by using the Pierce protein assay reagent (Pierce, Rockford IL. USA, cat. no.23225), in which protein content is measured in a reaction involving Cu^{2+} and bicinchoninic acid. BSA was used as a reference.

Phenolics determination

Explants were ground in liquid nitrogen and subsequently extracted in 80% ethanol. The phenolic content of this extract was determined using *p*-coumaric acid as a standard in an assay using the Folin-Ciocalteus phenol reagent (Hyodo et al. 1978).

Fatty acid composition

Fatty acid composition of neutral lipid and phospholipid fractions was determined by the method described by Hoekstra and van Roekel (1988).

Results

Growth and tissue composition

Upon incubation of bulb scale explants the fresh weight of the explants gradually increased (fig. 2.1); this was accompanied by a decrease in the dry weight percentage of the explant and a slight increase of the dry weight of all bulb scale explants (data not shown). Two significantly different ($p<0.0005$) groups of bulbs could be distinguished on account of different "vitality" of their explants. One group of bulbs yielded explants that reacted well to tissue culture (showing callus and shoot formation and no browning; mean explant score $\geq$ 2.5) while a second group of bulbs yielded explants with a low viability (no shoot formation, little callus formation and extensive browning) and a score $\leq$ 1.5 (fig. 2.2). If the explants were scored for only shoot formation, a direct correlation with our method of scoring was generally found

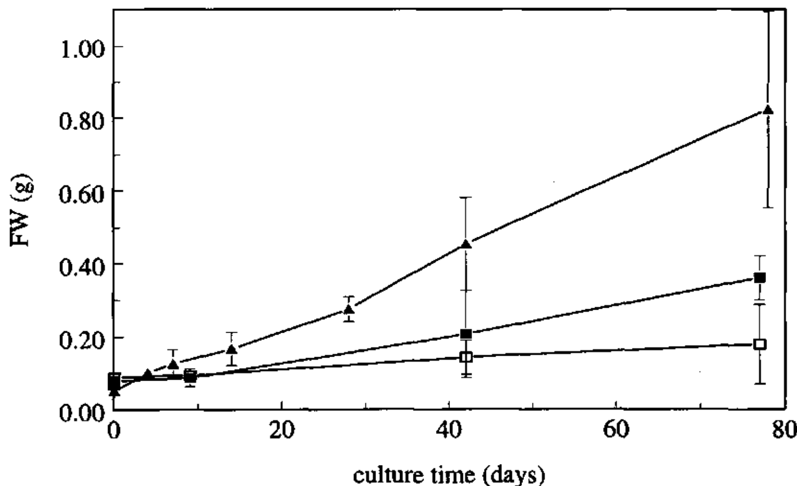

Figure 2.1 Changes in fresh weight in regenerating (■) and non-regenerating (□) tulip bulb scale explants and in regenerating tulip stalk explants (▲) during incubation. Values are mean ± SE. In case the error bars are not visible, they are smaller than the symbol size ($n\geq10$).

(data not shown). As can be expected, the regenerating explants showed a faster increase in fresh weight. (fig. 2.1). Stalk explants always showed good regeneration and a corresponding faster increase in fresh weight (fig. 2.1) in our experiments, so no difference between regenerating and non-regenerating stalk explants could be made.

In the phospholipid fraction of both groups of bulb scale explants, linoleic acid was the most abundant fatty acid: about 70% of the fatty acid of the phospholipids in the starting material comprised of this fatty acid (fig. 2.3). The fraction of linoleic acid

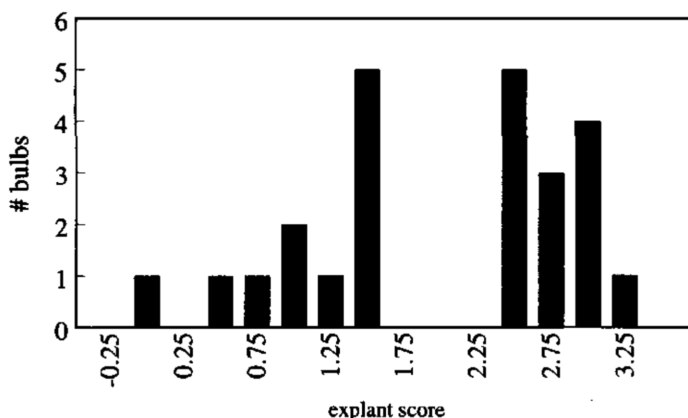

Figure 2.2 Frequency distribution of vitality scores of tulip bulb scale explants from 24 bulbs after 10 weeks of incubation. For each bulb the mean regeneration score was determined, from the regeneration score of six individual explants derived from this bulb, using the classification described in the material and methods section.

started to decrease upon incubation; already after 10 days it had reached a value of 55%, not changing appreciably afterwards. This was accompanied by an increase in the fraction of linolenic acid which increased from less than 1% to about 15%. The fractions palmitic (16:0), stearic acid (18:0) and oleic acid (18:1) did not alter upon incubation of the bulb scale explants (Table 2.1). Most of the changes occurred within the first 10 days after cutting the explant and were seen in both regenerating and non-regenerating bulb scale explants. No changes were observed in the first hours upon cutting (data not shown). After the changes over the first period, the composition remained stable throughout the culture period.

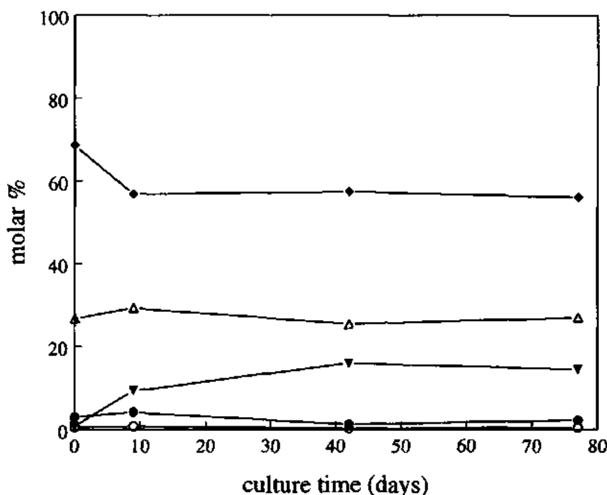

Figure 2.3 Changes in fatty acid composition of phospholipids in tulip bulb scale explants during incubation (representative example). Shown values are from regenerating scales, in non-regenerating scales the same values and changes are found. Legends: Δ: palmitic acid (16:0), O: stearic acid 18:0), ●: oleic acid (18:1),◆: linoleic acid (18:2) and ▼: linolenic acid (18:3).

In stalk explants, no appreciable changes in lipid composition were observed during incubation (Table 2.1). It can be also noted that at the onset of the incubation, the percentage of linolenic acid was much higher than in scale tissue. The fatty acid composition after 10 weeks of incubation was quite similar in scale and stalk explants, although the proportion of linolenic acid was still higher in the latter explant type. The total amount of phospholipids did not decrease during the incubation (data not shown).

Table 2.1 Fatty acid composition of the phospholipid fraction (molar %) of tulip explants after 10 weeks of incubation and of the starting material. Values presented are obtained from two representative experiments.

	Starting material		explants after 10 weeks of incubation		
Fatty acid	bulb scale tissue	stalk tissue	regenerating scale explants	non regenerating scale explants	regenerating stalk explants
16:0	26.7	25.3	26.9	27.1	28.2
18:0	0.6	0.3	0.4	0.7	0.6
18:1	3.0	3.7	2.2	3.7	6.3
18:2	68.6	46.8	56.0	57.3	47.1
18:3	0.8	24.5	14.4	11.1	17.9

In the neutral lipids of the bulb scale explants a very high fraction of linoleic acid was found (90%). This fraction decreased to about 75% upon incubation; also here a simultaneous increase in the fraction of linolenic acid was found (from about 1% to 17%: results not shown).

A very clear rise in the amount of extractable protein was observed during incubation of bulb scale explants: the amount of extractable protein increased from

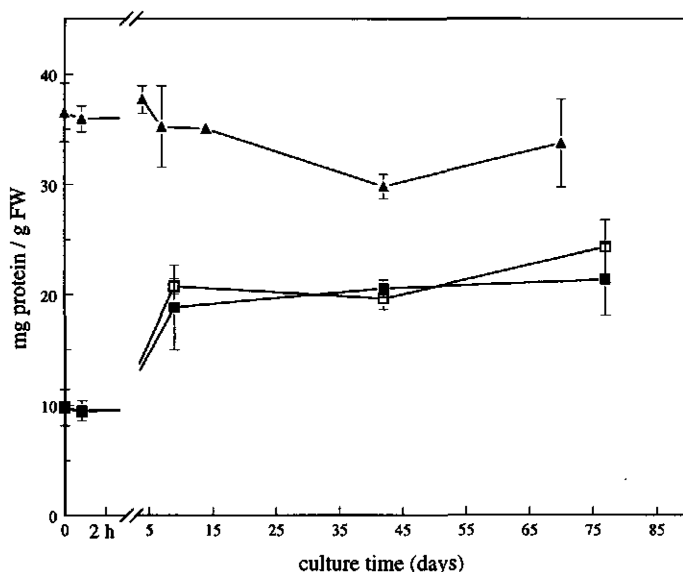

Figure 2.4 Changes in protein contents during incubation of tulip explants. Values are mean ± SD of four replicate determinations. For legends see figure 2.1.

about 10 mg/gram fresh weight in the original bulb scale tissue to about 20 mg/gram fresh weight within 10 days of incubation (fig. 2.4), stabilising afterwards. In stalk tissue, the amount of protein was higher (40 mg/g FW) at the start and it did not show such a marked increase.

Enzyme activities

Upon cutting the bulb scale explants, a number of rapid changes in activities of some of the measured enzymes were observed. Especially the activation of lipoxygenase upon cutting of bulb scale explants was rather striking: within the first hour already a doubling of the activity occurred (fig. 2.5A). This implicates a rapid response of the tissue. After the initial activation the LOX activity showed a decrease

in regenerating bulb scale explants, while in non-regenerating bulbs the LOX activity remained high and even tended to increase during incubation. The level of LOX activity was higher in stalk explants at the start of the incubation: in this system no clear increase upon cutting could be found, but the activity decreased to a low level during the incubation.

SOD activity in scale explants also showed a small increase immediately after cutting (fig. 2.5B), although to a much lesser extent than LOX. During the subsequent incubation, changes in activities in regenerating bulb scale explants and stalk explants occurred, although no clear trend in these changes could be distinguished. However, the activity in non-regenerating scale explants showed an increase. For stalk explants no activation of SOD was found upon cutting; the activities in this tissue were much higher than in the scale explants at the start of the incubation.

PAL activity showed an enormous increase in activity in the first week in all explant types; in regenerating bulb scale explants and stalk explants the PAL-activity then decreased, while in non-regenerating explants the activity of this enzyme remained at this high level (fig. 2.5C).

The activity of peroxidase gradually increased to a higher level than in the starting material in all three explant types that were tested (fig. 2.6A). For regenerating bulb scale explants this was followed by a decrease. For both the non- regenerating bulb scale explants and the stalk explants this increase continued for the remaining part of the incubation period of 10 weeks. A more or less comparable pattern was found for catalase, although the decrease in activity of the regenerating explants was less clear (fig. 2.6B). For stalk explants and non-regenerating bulb scale explants the catalase activity reached a rather high level after 6 weeks of incubation. Summarising, for both catalase and peroxidase a high level of activity was found for non-regenerating bulb scale explants in the final stages of incubation; still higher levels of activity were found in stalk explants, which generally showed extensive callus formation and regeneration.

The activity of PPO which is responsible for much of the visible browning increased gradually in bulb scale explants and reached a rather steady level after 6 weeks (fig. 2.6C). This steady level was higher for non-regenerating explants. In stalk explants, some transient increase might occur in the first 2 weeks, but the level which was eventually reached did not differ much from that in the stalk at the time of cutting

the explant. The amount of phenolics started to increase during the first week after cutting of the explants and remained at a high level in all explant types (fig. 2.6D). In stalks a temporary higher level was found, but this decreased to a level comparable to that found for bulb scale explants; no differences between regenerating and non-regenerating scales could be found.

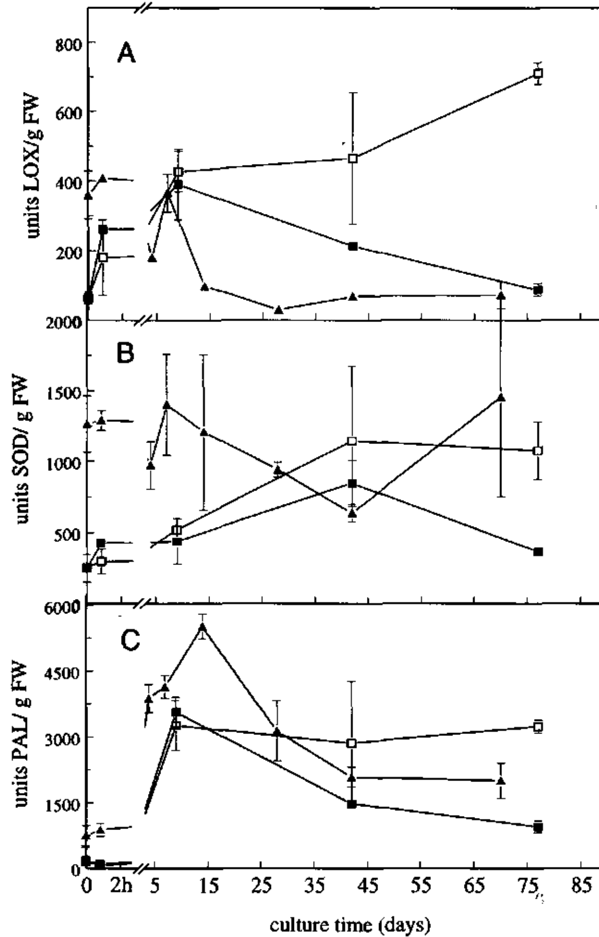

Figure 2.5 Changes in activity of LOX (A), SOD (B), and PAL (C) during incubation of tulip explants. Activities are calculated per gram fresh weight of starting material. Values are mean ± SD of four replicate determinations. For legends see figure 2.1.

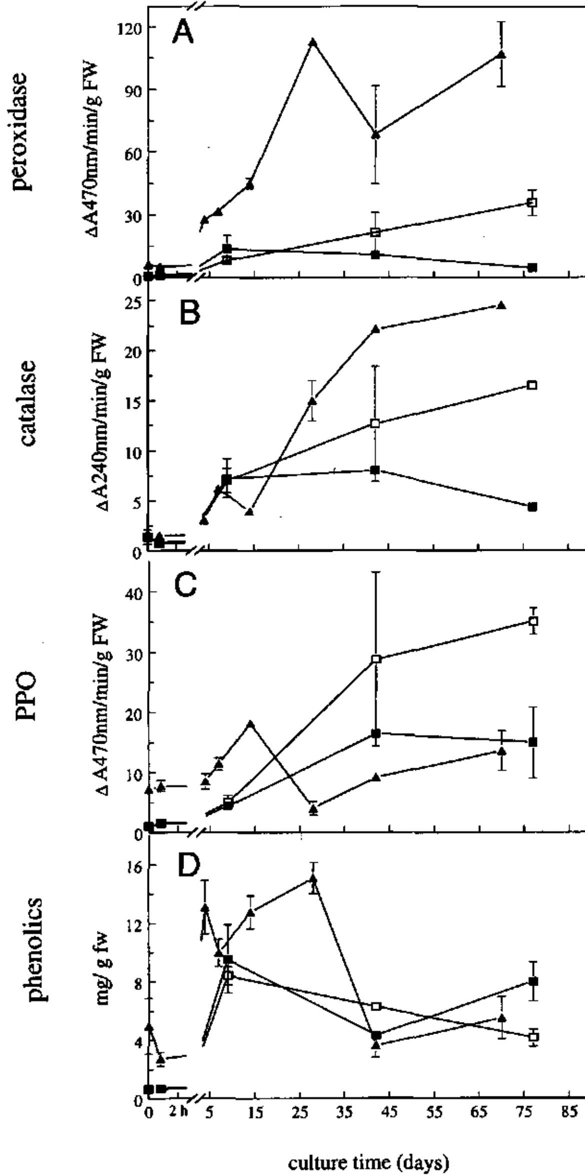

Figure 2.6 Changes in activity of peroxidase (A), catalase (B) and PPO (C) and changes in phenolic content during incubation of tulip explants (D). Activities are calculated per gram fresh weight of starting material. Values are mean ± SD of four replicate determinations. For legends see figure 2.1

Discussion

Two populations of tulip bulbs existed in our experiments which showed different reactions, i.e. bulbs yielding explants developing callus and shootlike structures ("regenerating") or showing loss of viability and almost no developmental reaction ("non-regenerating") when used as starting material for bulb scale based regeneration. In stalk explants only one group of explants could be found when using the same evaluation method. To evaluate the significance of changes in oxygen stress related processes for the developmental reaction of the bulb scale tissue, the behaviour of these two populations was compared to distinguish between regeneration related reactions and general responses of the tissue to either cutting or incubation. For the same reasons, tulip stalk explants which generally showed a good regeneration, were used as reference material.

The simultaneous decrease of linoleic acid and increase of linolenic acid in the lipid fatty acid fractions is in contrast with the expectation that the unsaturated fatty acids would decrease as a result of oxidative damage (Benson 1990; Dhindsa et al. 1996; Sung and Jeng 1994). As the total amount of phospholipids does not decrease, a net production of linolenic acid is apparently occurring. In the neutral lipid fraction of bulb scales, the proportion of linoleic acid (18:2) was very high (about 90%), meaning that at least two third of these lipids comprise of trilinolein (a lipid with a glycerol backbone containing three linoleic acid residues). Recently, this lipid has been reported to have an antioxidative function in animal systems (Chan et al. 1996). In these systems, a relation between the double bounds in natural lipids and its antioxidative capacity both in vivo and in vitro (by enhanced chemiluminescence) was shown. In this aspect, trilinolein proved to be an even beter antioxidant than trolox, able to reduce oxygen derived free radicals by almost 50%. The changes in fatty acid composition do not seem to be related to the developmental reaction of the tissue during incubation: similar changes were found both in regenerating and non-regenerating explants (Table 2.1). In stalk explants, no appreciable changes in fatty acid composition of the phospholipid fraction were found. Noticeably, the fatty acid composition in stalks is comparable to that found in the bulb scale explants after 10 weeks of incubation. This might indicate that this is the membrane fatty acid composition associated with growing tissue ; such a composition is already found in

stalk tissue at the start of incubation while the composition of scale tissue has to be adapted. Possibly the increase in linolenic acid is caused by the presence of a strong auxin (2,4-D) in the incubation medium (Goodrich and Travis 1995; Pastori and Trippi 1995). A fatty acid dehydrogenase (desaturase) might be activated; as a result, the fraction of incorporated linolenic acid will increase in both the phospholipids and the neutral lipids.

Also the increase in the amount of extractable protein in bulb scale explants is probably the result of a general response to cutting and subsequent incubation in vitro: adaptation of the tissue to its changed environment. Such an increase was also noticed in other tissue culture experiments (Benson et al. 1992b).

Two different phases can be distinguished in the activation of wounding stress-related enzymes in bulb scale explants. Immediately after cutting (the first phase), rapid changes are observed for some parameters. This is most clear for LOX; the activity in bulb scale explants doubles within the first hour. For bulb scale explants a small increase was also found for SOD. These increases are an indication for the production of free radical species and oxygen stress upon cutting the explants. No difference however, can be seen between regenerating and non-regenerating tulip explants.

Differences in reaction between the tested tissue types are primarily observed in the second phase of incubation, starting in the first week of incubation. Different patterns of changes in the tested parameters were observed (Table 2.2): 1) an increase

Table 2.2 *Summary of the pattern of reactions found during the second phase of incubation of stress related enzymes and substances in tulip explants expressed on a FW basis. Data derived from figures 2.5 and 2.6.*

Reaction type	non-regenerating scales	regenerating scales	regenerating stalks
1) increase	LOX, peroxidase PAL, catalase, SOD, and PPO	PPO	peroxidase and catalase
2) increase followed by decrease	phenolics	LOX, peroxidase, PAL, catalase, SOD and phenolics	PAL and phenolics
3) no large changes			SOD and PPO
4) decrease			LOX

during the incubation, sometimes reaching a steady level, 2) an initial increase followed by a gradual decrease, 3) no appreciable change during incubation and 4) only a decrease.

Most striking is the decrease in activity of LOX and PAL in the regenerating systems after their initial increase, while the activity of these enzymes remained high in the non-regenerating scale system. The higher LOX activity in the non-regenerating system indicates a continuous lipid peroxidation while the high PAL activity suggests also a continued stress reaction resulting in a continued production of phenolic substances (Davies 1972; Phillips and Henshaw 1977). This decrease in LOX activity indicates less lipid peroxidation in the regenerating explants. The activity is far lower in stalk explants than in scale explants during incubation; this might indicate a lower level of lipid peroxidation in stalk explants, possibly caused by a higher amount of antioxidative substances in stalk tissue. In all tissues, SOD activity is present. Although in non-regenerating explants a slow increase is found during incubation, no large differences between the tested tissues can be observed; this might indicate that the tissues do not suffer from the increased superoxide production during incubation and that they are capable to dispose of these radicals. On the contrary, activity of peroxidase and catalase increases and remains high in both regenerating stalks and non-regenerating scales while a decrease occurs in regenerating scales. This might be caused by the higher growth rate of the stalk explants, which leads to a higher metabolic rate resulting in the production of more hydrogen peroxide comparable to the production in non-growing, stressed scale explants. The higher peroxidase activity might also be related to the formation of new cell walls (Halliwell and Gutteridge 1985).

To get an indication of the changes in the processes directly involved in the browning process, the changes in the pool of monophenolics were followed in the course of incubation. The amount of phenolics increased enormously during the first days of incubation but showed no differences in the second phase; this might be caused by the fact that only monophenolic substances are measured; the visible browning is caused by polyphenolic substances produced by PPO from the monophenolics. Therefore, PPO activity might be a better marker for browning of the tissue. Indeed somewhat lower levels of PPO activity were found in regenerating scale

and stalk tissue, which did not show such a high degree of visible browning during incubation as non-regenerating scale explants.

The general higher activities of the oxygen stress related enzymes during incubation of scale explants, probably reflect a higher metabolic activity of the explant as a result of the changed physiological status caused by a different developmental pathway. The normal function of bulb scale tissue is the storage of nutrients (especially starch and fructans) for use by the plant in the following growth season. When bulb scale tissue is used as a starting material for regeneration purposes a developmental route has to be induced in which the metabolism is enhanced and the tissue has to redifferentiate. The redifferentiation of stalk material might be less drastic, as this tissue is already "programmed" for growth, because the material we used was actively growing at the moment we stored it at -2°C before use. This is also illustrated by our results; the enzymes most directly indicating undesirable oxidative processes (LOX and SOD) do not considerably increase upon cutting and/or incubation of stalk explants. So the scales derived from bulbs showing no satisfactory regeneration (part of the population in August, all bulbs in October), might have reached a point after which no dedifferentiation is possible and therefor wounding (e.g. cutting an explant) only results in death of the tissue. It has been suggested by Wernicke and Bretell (1980) that loss of genomic material is a result of programmed senescence in determinate organs. Such a reduction of genetic material, including parts which are necessary to enable regeneration, might have occurred in these ageing tulip bulb scales.

Differences found between regenerating and non-regenerating bulb scale explants might be the result and not the cause of the less favourable physiological reaction of the latter. The reactions of the non-regenerating bulb scale explants in the first phase of incubation after cutting, are generally identical to the reactions of regenerating bulb scale explants and also often of stalk explants. The observed changes in enzyme activity might be the result of the higher metabolic activity which is induced by the hormones in the medium independent of the developmental reaction of the tissue. Summarising, the problems of the *in vitro* propagation of tulip *via* bulb scale explants do not seem to originate from the lack of a system to cope with oxygen stress. The observed patterns in enzyme activity are rather similar in differently

reacting bulb scale explants and also comparable to the patterns seen in tulip stalk explants, that are known to show a satisfying regeneration reaction.

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Chapter 3

Adventitious regeneration from tulip, lily and apple explants at different oxygen levels

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Summary:

The effect of different O_2 levels on regeneration of adventitious organs was examined in various systems, *viz.*, shoot regeneration from tulip bulb scale and stalk explants, bulblet regeneration from lily scale explants and root regeneration from apple stem slices. The explants were exposed to 2% O_2 (low O_2), 100% O_2 (high O_2) or ambient air (20% O_2). Culturing under continuous high O_2 conditions had an moderate adverse effect in all systems. Continuous culture at a low level of O_2 had strong inhibitory effects in tulip and apple, but was promotive in lily. When low O_2 was applied only concurrent with the wound reaction, it had no or even an inhibitory effect (tulip stalks). An initial exposure to a high level of O_2 was examined in apple and had a (slight) inhibitory effect. Once the regenerated organs had been formed, low O_2 levels inhibited growth (in lily and tulip), and high O_2 levels were stimulating in tulip and inhibitory on organ growth in lily. Our results show that the allegedly toxic effect of O_2 is small or does not occur.

Introduction

The effect of O_2 on plant tissues has been studied frequently. Reactions of the reactive oxygen species with DNA, membranes or other cell constituents may result in severe damage and may cause inhibition of growth or even death (Ohlrogge and Kernan 1982; Halliwell and Gutteridge 1985). A relation between high O_2 levels and acceleration of senescence has been observed (Trippi et al. 1988; Scandalios 1990; Raven et al. 1994), whereas low O_2 inhibits senescence (Prince et al. 1981; Ohlrogge and Kernan 1982).

O_2 is an important component of the headspace of plant tissue culture containers. Its concentration may influence the growth of plant tissues cultured in vitro (Adkins et al. 1990). The effects of low O_2 in tissue culture have been summarised by Buddendorf-Joosten and Woltering (1994). O_2 may have significant effects during adventitious regeneration since oxygen radicals have a detrimental effect in wounded tissues (Farmer et al. 1994) and adventitious regeneration from plant tissues usually requires wounding. However, the effect of O_2 on regeneration has received little

attention. In adventitious shoot regeneration from leaf explants, improved shoot production has been observed by applying low O_2 levels just after excision of explants (Tanimoto and Harada 1983). To our knowledge, the effect of high O_2 levels on regeneration of adventitious organs has not yet been studied.

We have examined various regeneration systems at normal (20%), low (2%) or high (100%) O_2 concentrations. We hypothesized that during the wounding that occurs just after the explant has been excised, high O_2 conditions have an inhibitory effect because of the formation of high levels of oxygen radicals, while low O_2 levels during this period prevent this damage. When the wounding reaction has ended, high O_2 may promote and low O_2 reduce growth. Therefore, experiments were carried out in which high or low O_2 levels were applied continuously or transiently. The experiments were carried out with tulip bulb-scale explants since it has been suggested that in this case O_2-caused damage is a major obstacle (Koster, 1993). For comparison, we examined adventitious regeneration from tulip flower-stalk and lily bulb-scale explants. We also applied the three O_2 levels during adventitious root formation from apple stem slices because this system has been extensively examined, in particular in relation with the successive phases in the regeneration process (De Klerk et al. 1995).

Material and methods

Tulip

Plant material

Tulip bulbs (*Tulipa gesneriana* L.) cv. 'Apeldoorn' were grown in the field and harvested early July. After drying, they were stored at 30°C for at least two weeks.

Tulip bulb scale explants

Bulbs from which the brown skin had been removed, were longitudinally cut into four equal parts. After rinsing with 70% (v/v) ethanol (30 sec) and water (30 sec), the bulb parts were sterilized in 1% (w/v) hypochlorite solution for 30 min and rinsed three times with sterile water. The second and third outermost scales were used as starting material. Explants (2.5 mm thick, 70-100 mg) were cut from the basal part of the scale just above the basal plate. Only the lower two explants were used. So from

each bulb, 16 explants were cut. They were cultured in Petri dishes with the basal side down on MS medium (Murashige and Skoog 1962) with 3% sucrose, 0.5 g/l casein hydrolysate (Gibco), 0.1 g/l myo-inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 1 mg/l 2,4-D, 1.5 mg/l BAP and 6 g/l agar (BBL) (Gude and Dijkema 1997). The pH was adjusted to 6.0 before autoclaving. The Petri dishes (Greiner 604102: 10 x 2 cm) had a lid with mounts and were not sealed to provide optimal exchange between the headspace and the atmosphere outside the Petri dishes. The explants were cultured for 10 weeks at 20°C in the dark, either under atmospheric O_2 conditions or in aeration chambers (void volume 40 L). After an initial strong flush to acquire the desired atmospheric condition they were flushed with 2% O_2-98% N_2 or 100% O_2 (10 $mL.min^{-1}$). Also the effect of incubation in 2% O_2 for 3 days, or 1, 2, 4 or 6 weeks followed by transfer to atmospheric O_2 conditions was investigated.

Experiments were carried out in July-August, when regeneration is satisfactory, and in October when the regeneration potential is small (Koster 1993).

Tulip stalk explants

Bulbs were stored at 20°C until the middle of October and then for one month at 17°C. After that, the bulbs were planted in pots with soil and stored at 9°C for 6 weeks. During this period, rooting and some sprout development occurred. After this treatment, the pots were stored at -2°C until use. Experiments were performed in January-February. The stalks were isolated from the bulbs, the top was removed and the stalks were surface-sterilised as described for the bulb scale explants. Six explants per stalk were cut around the nodes. The explants were incubated for 10 weeks on MS medium as described for bulb scale explants.

Evaluation of explants

After 10 weeks, callus and shoot formation and browning of the explants were scored according to the following classifications: little callus formation (1 point), extensive callus formation (2 points), presence of adventitious buds (2 points) or presence of shoots (4 points). For browning, a negative score was assigned to the explant (-1 point). Each explant was individually scored, and the mean score for the explants derived from one bulb was calculated.

Lily

Bulbs of *Lilium longiflorum* "Snow queen" were stored after harvest at -1°C in peat until needed. After sterilising the scales, explants were cut (7x7 mm) and incubated, on medium as described by Djilianov et al. (1994) at 20°C in the dark for 10 weeks. The explants from one bulb were distributed over various Petri dishes and subsequently incubated under atmospheric conditions or in aeration chambers that were flushed with 2%O_2 / 98% N_2, or 100% O_2 (void volume 40 L, flushing rate 10 mL/min). Also experiments were performed in which the explants were incubated in 2% O_2 immediately after cutting for 3 days, or 1, 2, 4 or 6 weeks and after that, transferred to atmospheric O_2 conditions. The explants were incubated at 20°C and evaluated after 10 weeks. In this evaluation, we scored the number and weight of bulblets and leaves that were formed on the explants.

Apple

Apple (*Malus*) 'Jork 9' microcuttings were propagated and 1-mm stem slices were cut and cultured as described by De Klerk et al. (1995). The slices were cultured at 20°C under atmospheric conditions or in aeration chambers that were continuously flushed with 100% O_2 or 2% O_2 / 98% N_2 (void volume 40 L, flushing rate 10 $mL.min^{-1}$). Also exposures of 24h to 100% or 2% O_2 were examined. These exposures were given at various times after cutting. The explants were kept under atmospheric conditions before and after the exposure to high or low O_2. After 20 days, the number of roots formed on the explants was determined and the number of explants without roots was counted. For each treatment, three Petri dishes with 30 slices per dish were used.

Results

Tulip

In regeneration of tulip bulb scale explants, different stages can be observed: two weeks after cutting the explants the adaxial and abaxial epidermes started swelling, later forming callus; 4-6 weeks after cutting, adventitious buds started to

appear on the upper cut surface of the explants. Shoots were formed 8-10 weeks after cutting.

Table 3.1 Vitality of tulip scale explants after 10 weeks of incubation at either continuous normal, low or high O_2 or after transfer from low O_2 to normal O_2 conditions after the indicated periods. Values are mean ± SE and derived from at least 10 different samples (10 bulbs, 50-60 explants).

		Vitality score per explant
July-August series		
2 %		-0.1 ± 0.1
20 %		1.9 ± 0.3
100 %		0.6 ± 0.1
October series		
2 %		-0.4 ± 0.1
20 %		-0.3 ± 0.1
transfer 2 to 20%	after 3 days	-0.1 ± 0.1
	after 1 week	-0.5 ± 0.1
	after 2 weeks	-0.4 ± 0.1
	after 4 weeks	-0.5 ± 0.1

Scales from tulip bulbs cut in July-August (shortly after harvest) showed satisfactory regeneration and a reasonable vitality under atmospheric conditions (20% O_2, table 3.1). High (100%) or low (2%) O_2 conditions had a negative effect on vitality (both reduced callus and shoot formation; Table 3.1). The vitality score

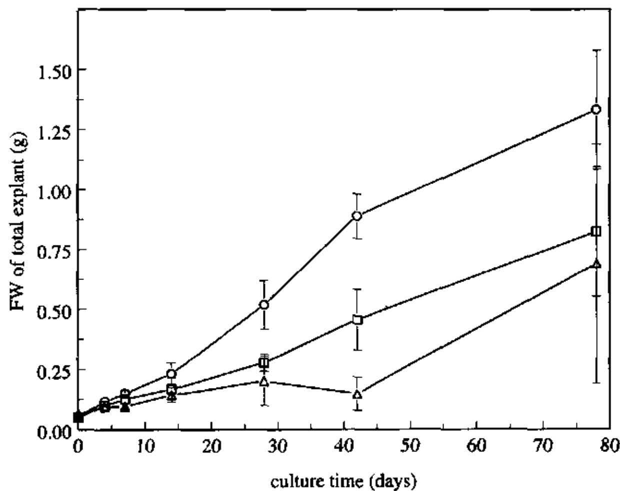

Figure 3.1 Changes in fresh weight of tulip stalk explants incubated in normal (20%: □:), high (100%: O) and low O_2 (2%: Δ). Values are average ± SE. (n ≥ 10).

Table 3.2 Vitality of tulip stalk explants and number of shoots regenerated from the explants after 10 weeks of incubation at either continuous normal, low or high O_2 or after transfer from low O_2 to normal O_2 conditions after the indicated periods. Values are mean ± SE and derived from at least 8 samples (24 explants).

		Vitality score per explant	Shoots per explant
2 %		1.0 ± 0.6	0.8 ± 0.7
20 %		3.7 ± 0.5	6.1 ± 0.9
100 %		2.8 ± 0.5	3.0 ± 0.6
transfer 2 to 20%	after 3 days	3.4 ± 0.4	2.3 ± 0.3
	after 1 week	2.7 ± 0.5	1.5 ± 0.3
	after 2 weeks	2.4 ± 0.4	1.0 ± 0.2
	after 4 weeks	2.2 ± 0.5	1.2 ± 0.3

correlated closely with the regeneration of adventitious shoots from the explants (data not shown). As expected (Koster 1993), the explants from bulbs cut in October and cultured at 20% O_2, all had a very low vitality. From these explants, no shoot formation occurred and only some callus formation and small adventitious buds were observed. At low O_2, the score did not improve. Because O_2 may have a damaging effect only during the wounding reaction, and after that a promotive effect, we applied low O_2 for limited periods just after cutting. Both vitality score and regeneration did not improve significantly (Table 3.1).

Continuous incubation of stalk explants of tulip under the three O_2 regimes led to a comparable response as in the bulb scales, but vitality and the numbers of regenerated shoots were much higher (Table 3.2). The fresh weight per shoot formed on the stalk explants at 100% O_2 was higher than under 20% O_2 (33±12 vs. 22±3 $mg.shoot^{-1}$ respectively) whereas fresh weight was lowest at 2% O_2 (17±3 $mg.shoot^{-1}$). During the culture period, the explants showed a considerable increase of fresh weight that was also largest at 100% and lowest at 2% O_2 (Fig. 3.1). In stalk explants, an exposure to low O_2 just after cutting, reduced both the vitality and number of regenerated shoots with the duration of exposure (Table 3.2). This indicates that the application of low O_2 conditions had a very marked negative effect in the early phases of the tissue culture process.

Apple

In the apple stem-slices, the percentage of explants that did not form roots was higher at 2 or 100% O_2 than at 20% ($P < 0.001$ or $P < 0.005$, respectively; table 3.3).

Table 3.3 Percentage of apple stem slice from which no roots had regenerated during different O_2 treatments.

treatment	2% O_2	20% O_2	100% O_2
continuous	12.5	2.2	6.7
0-24 h	4.4		13.3
24-48 h	2.2		7.8
48-72 h	3.3		1.1
72-96 h	1.1		0
96-120 h	2.2		2.2

Pulses with 2% O_2 for 24 h did not change the rooting percentages significantly, but 24h pulses with 100% O_2 from 0-24h or 24-48 h increased the percentages of non-rooting slices significantly ($P< 0.001$ or $P< 0.005$, respectively). The number of roots per rooted slice showed similar tendencies, but significantly less root numbers were achieved only at the continuous low O_2 incubation (Table 3.4).

Table 3.4 Number of roots formed on apple stem slices cultured at different O_2 treatments. Only the numbers of roots formed on root-forming explants were included in the calculation (mean ± SE).

treatment	2% O_2	20% O_2	100% O_2
continuous	3.6 ± 0.4	5.3 ± 0.3	4.7 ± 0.2
0-24 h	4.5 ± 0.4		4.1 ± 0.3
24-48 h	4.8 ± 0.2		4.4 ± 0.1
48-72 h	4.6 ± 0.2		4.8 ± 0.5
72-96 h	4.3 ± 0.4		5.6 ± 0.2
96-120 h	4.8 ± 0.2		4.7 ± 0.3

Lily

In lily, the number of bulblets regenerated under low O_2 was considerably higher (Fig. 3.2A), whereas no significant difference between high and normal O_2 occurred. When explants were exposed to low O_2 for a period up to 4 weeks after cutting and then transferred to normal O_2, no significant effect of the low O_2 conditions was observed: treatments of 3 days to 4 weeks with low O_2 all lead to reactions comparable to those of the controls incubated in 20% O_2.

Incubation under different O_2 regimes affected the fresh weight of these bulblets in different ways. The mean fresh weight per bulblet both under high and low

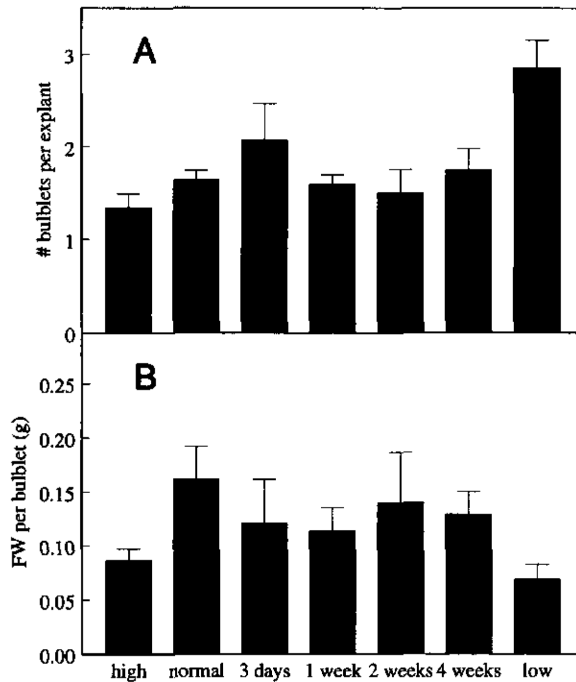

Figure 3.2 Number (A) and fresh weight (B) of bulblets formed on lily scale explants after 10 weeks of incubation in normal, high or low O_2 or after transfer from low to normal O_2 after 3 days, 1, 2 or 4 weeks. Values are average $\pm$ SE. (n $\geq$ 10).

O_2 was slightly lower than under normal, atmospheric conditions (Fig. 3.2B), while short exposures to low O_2 had no clear effect on fresh weight.

Discussion

It is believed that high concentrations of O_2 are toxic for plant tissues (Trippi et al. 1988; Raven 1994; Scandalios 1990). Indeed, continuous incubation at a high level of O_2 in the headspace of tissue culture containers led to a reduction of the regeneration of the explants in all four tissue culture systems that were examined. In

apple, the number of explants which did not regenerate roots increased while the number of regenerated roots showed a tendency to decrease. In lily, the number of regenerated bulblets was slightly lower during incubation at high O_2. In tulip explants a reduction of vitality was observed, and in stalk explants a reduction of the number of shoots was found. It should be noted, however, that inhibition was only moderate and in none of the systems O_2 appeared to be very toxic.

Incubation at low O_2 resulted in reduced regeneration in tulip and apple, whereas regeneration from lily scale explants was enhanced. Remarkably, in lily continuous low O_2 resulted in more bulblets than incubation for four weeks at low O_2 followed by six weeks of incubation under normal O_2. This might be caused by a combination of two effects at low O_2: a positive effect on the number of initiated bulblets and a negative one on the rate of this initiation. As a result the formation of the primordia might not be completed after four weeks resulting in the formation of more primordia under continuous low O_2.

Altered O_2 levels had a different effect on the growth of the regenerated structures once they had been formed. In tulip, growth of the regenerated shoots at high O_2 was faster and at low O_2 slower. The large increase in fresh weight at 100% O_2 conditions is comparable to a considerable fresh weight increase found in potato callus grown in a 70% O_2 environment promoted growth expressed as fresh weight or dry weight increase (Van der Plas and Wagner 1986) and the enhancement of the plating efficiency of rice, tomato and jute protoplasts was enhanced by a 100% O_2 pulse (d'Utra Vaz et al. 1992). In both cases, enhanced growth was attributed to improved O_2 availability. We suggest that an improved oxygen diffusion causes improved growth also in tulip.

The opposite effects of high O_2 levels on adventitious regeneration and on the growth of regenerated organs indicates that the effect of O_2 depends on the physiological state of the plant tissue. We presumed that during the wounding, occurring after excision of the explant, high O_2 conditions have an inhibitory effect because of the formation of high levels of oxygen radicals, and that low O_2 conditions promote regeneration during this period. When the wounding reaction has ended, high O_2 is supposed to promote and low O_2 to reduce growth. Therefore, experiments were carried out in which the high or low O_2 levels were applied only for a short period.

For regeneration of roots from apple stem slices, the timing of the successive phases in regeneration has been established (De Klerk et al. 1995). Therefore, we studied the effect of transient exposures to low or high O_2 most extensively in apple stem slices. The formation of the root primordium starts 24h after excision of shoots or stem slices (De Klerk et al. 1995; Jasik and De Klerk 1997). High O_2 had a small, but significant inhibitory effect when applied during the initial 24h of culture. This coincides with the period of dedifferentiation and with the O_2 wounding reaction suggesting a damaging effect of high O_2 during this period.

For tulip explants, the timing of the different phases of the regeneration may be derived from histological examinations (Wilmink et al. 1995; Koster 1993; Chanteloube et al. 1993). Dedifferentiation occurs in the first two weeks after cutting the explant and the formation of meristems is completed after another 2 weeks. Low O_2 was inhibitory and not -as expected- promotive when applied during this period. One of the most striking results in the present research is the strong decrease in the number of shoots per stalk explant observed after a 3 day incubation at 2 % O_2 and subsequent incubation for 9 weeks at normal O_2 (table 3.2; from 6.1 to 2.3). Low O_2 resulted, both for scale segments and for stalk explants in a reduced regeneration performance.

Our results with apple and tulip are in contrast with the results of Imamura and Harada (1981) and Tanimoto and Harada (1983) who report a positive effect of a low O_2 (0-5% O_2) pulse in the first phase of incubation of tobacco pollen and *Torenia* stem fragments, preceeding embryogenesis and bud formation respectively. These differences cannot be explained by the place in the explants where the regeneration starts: in apple it occurs in the fourth cell-layer from the cutting surface (Jasik and De Klerk 1997), while in *Torenia*, tulip scale and stalk explants the regeneration starts also just below the surface of the explants (Tanimoto and Harada 1981; Koster 1993; Chanteloube et al. 1993; Wilmink et al. 1995). The difference in reaction might be caused by differences in the physiological state of the starting tissue, enabling the tested systems to cope more efficiently with the oxidative stress of the tissue culture process than tobacco or *Torenia* or might be caused by a too low level of reactive oxygen species. Some authors have described a role for the transient presence of such reactive oxygen species in the initiation of regeneration (Burdon, 1996; DeMarco and

Roubelakis-Angelakis 1996; Suzuki et al. 1997), apart from the general damaging effects of such oxygen radicals (Halliwell and Gutteridge 1985).

In summary, we conclude that the allegedly inhibitory effect of O_2 during regeneration does not occur or was only small in regeneration from tulip scale and flower stalk explants, lily scale explants and apple stem slices. In fact, a short initial period at low O_2 even reduced the number of regenerating shoots in tulip stalk explants. Once the adventitious structures have been formed, an elevated O_2 level might even promote growth. Apparently, O_2-stress is not the direct cause of the decreased viability and unsatisfactory regeneration performance in this type of explants.

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Chapter 4

Effects of growth conditions on tulip bulb scale performance during micropropagation

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Summary:

In order to improve the regeneration performance of tulip bulb scale explants during micropropagation the influence of a number of antioxidative substances was added to the incubation medium. The effect on tissue culture performance and on the activity of a number of oxygen-stress related enzymes (SOD, lipoxygenase, catalase and peroxidase) was measured as well as the production of phenolic substances and TBArs. In addition we tested the use of anti-oxidants during the cutting of an explant submerged in a medium containing these components.

In all cases the regeneration of adventitious shoots or buds on the explants could not be clearly stimulated by the additions, although the vitality could be slightly improved by the addition of trolox, glutathione, catalase, BHT or oxalic acid. However, this improvement of vitality did not lead to a better regeneration and had no clear effect upon the activation of stress related enzymes. Submerged cutting resulted in a transient lower TBArs production directly after cutting, but upon incubation no differences with the control treatment could be found. Therefore we concluded that the antioxidative status of bulb scale tissue might be sufficient to cope with oxygen stress during cutting and incubating of explants and it does not seem likely that the poor regeneration of tulip bulb scale explants is caused by an extreme sensitivity to oxidative stress.

Introduction:

Tissue culture leads to a stress situation for plant tissues: cutting explants implicates wounding of the tissue and incubation on nutrient media establishes a physiological state which differs from the situation *in planta* with respect to salt composition and osmolality. Also the gaseous atmosphere is different during incubation of the explants, generally comprising higher oxygen concentrations and often accompanied by ethylene production. Free radicals, especially reactive oxygen species (e.g. superoxides and hydroxyl radicals) are produced during the preparation of an explant for tissue culture, a situation which is comparable to processes during pathogen attack or senescence. This leads to a wide range of reactions causing effects

like lipid peroxidation (membrane damage) and damaging of DNA and structural proteins. In lipid peroxidation also the activity of lipoxygenase is important: this enzyme catalyses dioxygenation of unsaturated fatty acids, producing fatty acid hydroperoxides, short chain aldehydes and jasmonic acid and has been implicated with senescence and loss of vitality (Sung and Jeng 1994; Kumar and Knowles 1993). It is not clear if LOX can only peroxidize free fatty acids either liberated from the glycerol backbone of the phospholipid by phospholipase A_2 activity (De Vos et al. 1993; Leshem et al. 1994; Zhuang et al. 1996) or after free radical attack of the membrane phospholipids (Benson et al. 1992; Benson and Roubelakis-Angelakis 1992). Some authors suggest that peroxidation of fatty acid may occur in the membrane; the peroxidized fatty acids are then removed by phospholipase A_2 (Schraudner et al. 1997). Some of the breakdown products that are formed may function as signal molecules such as ethylene and jasmonic acid, which are both able to induce further damaging reactions or induce the wound healing response (Benson 1990; Biddington 1992; Rakwal et al. 1996). Ethylene can also be produced via an enzymatic route (Benson 1992) and can have both positive and deleterious effects on the tissue, depending on the system (Adkins et al. 1990).

Some of the processes involved are shown in figure 4.1. As a result, a wide variety of defence mechanisms is activated comprising e.g. a number of enzymes (SOD, catalase and peroxidases) able to process the free radicals into non-damaging compounds and the production of a number of antioxidative substances (such as vitamin E and gluthatione) which can quench reactive oxygen species.

The application of different tissue culture conditions can be used to improve performance of recalcitrant plant tissues in tissue culture (Chauchan et al. 1992; Ishii 1988; Saleem and Cutler 1987). These may comprise the alteration of type, concentration or combinations of hormones (Ernst 1994; Leshem et al. 1994; Saftner and Wyse 1984), or the use of media differing in osmolality or salt composition. The medium additions can be very diverse and sometimes not very well characterised; often a mixture of antioxidants is used (coconut milk in plant tissue culture or foetal calf serum in animal and sometimes in plant systems, Van der Maas et al. 1993) but also specific antioxidative substances are often used to improve the vitality of the tissue (Flores et al. 1994; Kuroda et al. 1991). The better performance of tissues in the

presence of antioxidants is thought to be the result of an improved ability to process e.g. oxygen free radicals (Dalton and Morris 1993).

The regeneration of shoots on tulip bulb scale explants is very cumbersome, resulting in severe browning and low viability of the explants, suggesting a high sensitivity for oxidative damage. To test this hypothesis, a number of well-defined antioxidative species such as trolox, a watersoluble vitamine E analog (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Wu et al. 1993), butylated hydroxy toluene (BHT), a very potent antioxidant (Flores et al. 1994), and caffeic acid, an inhibitor of lipid peroxidation (Devasagayam et al. 1996) were applied to improve regeneration of shoots on tulip bulb scale explants. Furthermore, substances with a general antioxidative function were added like oxalic acid (Dalton and Morris 1993), salicylic acid (Creemers-Molenaar et al. 1992), and gluthatione (De Vos et al. 1994). Paclobutrazol which has been described to have positive effects on the anti-oxidative functioning of tissues (Kraus et al. 1995; Lurie et al. 1994) and on regeneration (Leshem et al. 1994; Lilien-Kipnis et al. 1994) was also tested as well as the addition of catalase and peroxidase to the medium. Inhibiting lipoxygenase activity by the addition of phenidone (1-phenyl-3-pyrazolidone), (Krens et al. 1994; Farmer et al. 1994) might also affect the production of ethylene and jasmonic acid. Jasmonic acid, which may function as signal molecule in activating the oxygen stress defence mechanisms leading to improved regeneration (Farmer and Ryan 1994, Rakwal et al. 1996), was also tested itself. Blockers of ethylene synthesis may provide better results in tissue culture performance (Biddington 1992; Roustan et al. 1992) since ethylene is related to wounding, oxygen stress and browning (Housti et al. 1992). Influencing the ethylene metabolism (e.g. by adding AVG) or the response of the cells to ethylene might improve the performance of the explants (Biddington 1992). Figure 4.1 gives a schematic mode of action of most of the mentioned compounds.

Since the most drastic oxygen stress is to be expected at the moment of cutting, submerged preparation of the explants might improve their performance due to the reduced oxygen levels in aqueous solutions. Preliminary positive effects of submerged cutting on TBArs production were shown before (van Rossum and van der Plas, 1995). Addition of antioxidative substances to the solution during cutting might further improve tissue performance: therefore, the effect of trolox and phenidone was tested. To reduce the amount of radicals produced by the Fenton reaction in this phase,

a specific iron chelator, detapac (diethylenetriaminepentaacetic acid) was used to chelate the Fe-ions necessary in this reaction (Ohba et al. 1995).

To characterize the effects of the various compounds on the performance of the bulb scale explants in tissue culture, we monitored a number of physiological changes such as fatty acid composition of the phospholipids, the production of phenolic substances which cause browning of the explants or can be toxic to the tissue itself (Hyodo et al. 1978; Housti et al. 1992), production of TBArs as a indicator for oxidative stress and the activation of peroxidase, catalase, SOD, and lipoxygenase. The latter enzymes may be involved in the stress response during incubation of regenerating and non-regenerating bulb scale explants.

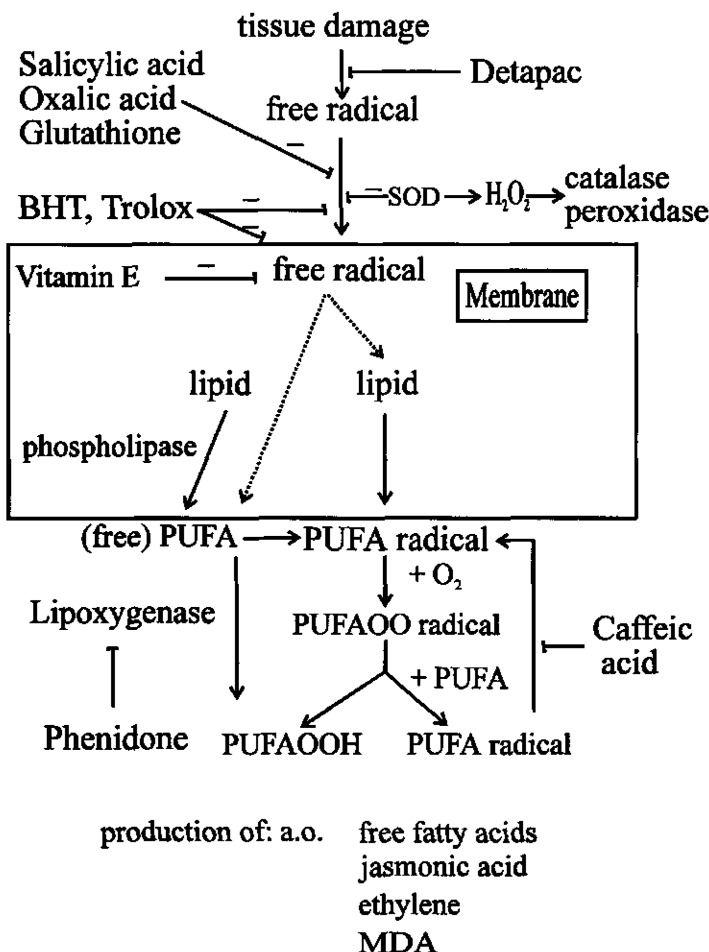

Figure 4.1 Schematic overview of oxygen-stress related processes and products. Also the supposed action mechanism of the additives used in the experiments described in this chapter are shown. A number of these processes will occur at the boundary of membrane and cytoplasm.

Material and methods

Tulip material

Tulip bulbs (*Tulipa gesneriana* L.) cv. Apeldoorn were field grown and harvested in early July. After drying they were stored at 30°C for two to four weeks. Immediately after this period explants were cut (end of July / August).

Tissue culture

After removal of the brown skin, bulbs were longitudinally cut in four parts. After rinsing first with 70% ethanol and then with water to remove remaining ethanol (each 30 seconds), the bulb parts were sterilised in 1% hypochlorite solution for 30 minutes. After sterilising, the bulbs were rinsed three times in sterile water. The second and third outermost scales were used as starting material. The explants were cut from the basal part of the scale, just above the basal plate. Explants were ± 2.5 mm thick and weighed 70-100 mg. Explants were put on the medium with the basal side down and grown on full-strength MS medium (Murashige and Skoog 1962), containing 3% sucrose, 0.5 g/l casein hydrolysate (Gibco), 0.1 g/l myo-inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 1 mg/l 2,4-D, 1.5 mg/l BAP and 6 g/l agar (BBL) (Gude and Dijkema 1997) ; the pH was adjusted to 6.0 prior to autoclaving the medium. The explants were cultured at 20°C in the dark.

Sample preparation

Samples were ground in liquid nitrogen and extracted in 1 ml of buffer containing 0.05 M potassium phosphate buffer pH 7.0, 1 mM $CaCl_2$, 1 mM KCl and 1 mM EDTA (Benson et al. 1992). The resulting extract was used for enzyme and MDA determinations.

MDA- determination

The amount of MDA was estimated by determining the amount of thiobarbituric reactive material. This was done by heating the material in 0.25% TBA in 10% trichloroacetic acid for 30 minutes. MDA equivalents were determined using the absorbance of the MDA-TBA complex at 532 nm corrected with the non-specific absorbance at 600 nm.

Phenolics determination

Explants were ground in liquid nitrogen and subsequently extracted in 80% ethanol. The phenolic content of this extract was determined using *p*-coumaric acid as a standard in an assay using the Folin-Ciocalteus phenol reagent (Hyodo et al. 1978).

Protein content

Protein content of the samples was determined by using the Pierce protein assay reagent (Pierce, Rockford IL. USA, cat. no. 23225), in which protein content is measured in a reaction involving Cu^{2+} and bicinchoninic acid. BSA was used as a reference.

Enzyme determinations:

Catalase activity was determined by incubation of 20 µl of the enzyme extract in 1 ml of 0.1 M potassium phosphate buffer pH 7.0 containing 27 mM of hydrogen peroxide. The disappearance of hydrogen peroxide was spectrophotometrically measured at 240 nm in a thermostated spectrofotometer (Shimadzu UV1601PC) at 30°C (Benson et al. 1992).

Peroxidase activity was determined spectrophotometrically by measuring the rate of increase in oxidised guaiacol at 470 nm in a thermostated spectrophotometer at 30°C using 15 mM guaiacol in 0.1 M phosphate buffer pH 5.0 and 27 mM hydrogen peroxide (Benson et al. 1992).

Lipoxygenase activity was determined by measuring the formation of conjugated dienes at 234 nm using 10 mM linoleic acid as the substrate in a thermostated spectrophotometer at 30°C (Sanz et al. 1992).

Superoxide dismutase activity was determined by a modified method according to Beyer et al. 1987. In this method, the inhibition of reduction of NBT (nitro blue tetrazolium) by enzyme extracts is measured. A dilution series of the enzyme extract (2, 4 and 8 times diluted) was pipetted into a microtiter plate. The plate also contained wells without extract and with either 1 or 10 units commercially available SOD (Sigma; 1 unit is defined as the amount of enzyme capable of 50 % reduction of cytochrome c in a coupled system with xanthine and xanthine oxidase; in our system this is identical to a 50 % inhibition of total SOD-inhibitable NBT-reduction). To all wells, 0.2 ml of a solution containing 0.05 M potassium phosphate

buffer pH 7.8, 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA and 0.025% triton X100 was added. To start the reactions 20 μl of 10 mM riboflavin was added, at the same time placing the plate under fluorescent light for 1.5 minutes. After this period the microtiter plate was transferred to the dark, riboflavin was added to the wells containing the blank, and absorbance was determined at 630 nm. SOD activity was determined by calculation of the amount of extract which gave 50 % inhibition of NBT-reduction.

Tissue culture additions

All additions were filter sterilized and added just before the medium was poured into the petri-dishes. Trolox (Aldrich) was added to the culture medium at a concentration of 10 μM; BHT (butylated hydroxytoluene), caffeic acid, jasmonic acid and salicylic acid were used in concentrations of 50 μM; glutathione was tested in a concentration of 1 μM, oxalic acid in a 5 μM concentration. AVG (2-aminoethoxy vinyl glcyine) was added in a concentration of 30 μM and paclobutrazol in a concentration of 3 mg/l. Two different enzymes were added to the medium: peroxidase (Horse radish, type I, Sigma, 50 mg/l) and catalase (Serva, 130 units/l). Foetal Calf serum (Serva) was used at a concentration of 17.5 % (v/v) in the tissue culture medium.

Phenidone and detapac (both from Sigma) were applied during submerged cutting of the explants at a concentration of 0.1 mM in the cutting medium, trolox was used at a 10 μM concentration. The cutting medium was similar to the medium used for incubation, but without agar.

Differences in developmental reaction between explants derived from different bulbs may be large as shown previously (chapter 2). Therefore, each treatment existed of at least 20 explants cut from at least 3 bulbs.

Results:

Changes during standard incubation conditions:

Upon incubation of tulip bulb scale explants a number of different reactions could be observed. MDA content (measured as TBArs) increased very rapidly upon

cutting and incubation and production of phenolics was observed after about 1 week (fig. 4.2). Furthermore clear changes in fatty acid composition of the phospholipids were observed; most markedly a decrease in linoleic acid (from 70 to 55) and an increase in linolenic acid (from 1 to 15%) occurred (data not shown, chapter 2).

In the explants, increases in activity of catalase, peroxidase, SOD, and lipoxygenase were observed during incubation. The increases in LOX activity and, to a lesser extent, SOD activity occurred within 1 day after cutting the explants and seemed wounding related. After about one week of incubation an increase of catalase and peroxidase was observed in all tested explants. In well-regenerating explants, all

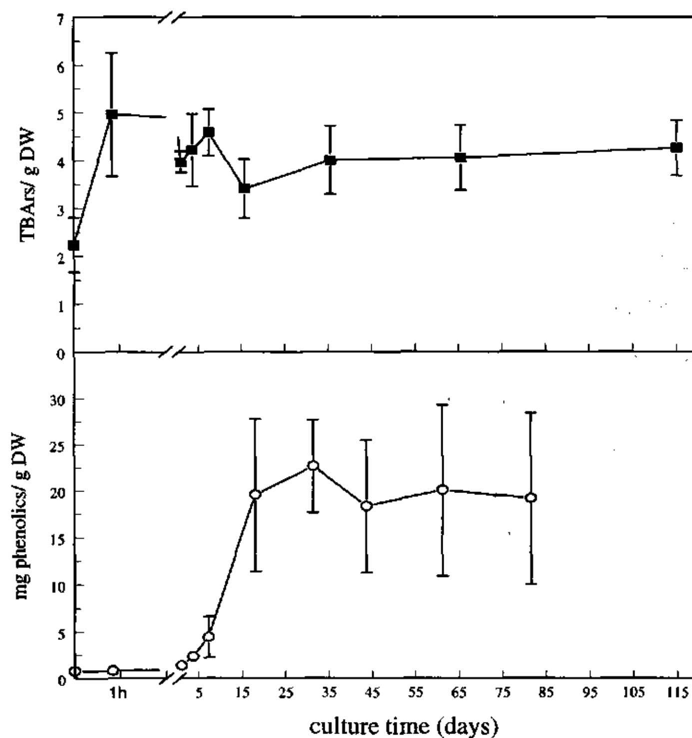

Figure 4.2 Changes in the amount of TBArs and phenolic substances during standard incubation of tulip bulb scale explants. Values are mean ± SD. Each data point is derived from at least 4 separate determinations.

these activities decreased again after about two weeks in contrary to non-regenerating explants in which the activity remained at a high level (chapter 2).

Effect of various additions to the tissue culture medium

In the series of experiments described in this chapter, often no full regeneration (shoot formation) was observed, irrespective of the treatment. However, differences in reaction of the tissue were observed with respect to swelling of the tissue, callus formation, start of adventitious bud formation or the degree of browning. Therefore, the percentage of explants forming callus was used as a method to evaluate the performance of the tissue during the treatment. In general, the percentage of callus forming explants correlated very well with the visible appearance of the explants in the different treatments (swelling of explants and/or (lack of) browning, results not shown). The different additions to the tissue culture medium were tested in a number of experiments, each with an independent control i.e. explants cut from the same bulbs but cultured on standard medium.

The addition of antioxidative substances generally did not significantly improve the percentage of explants forming callus; however for a number of additions (trolox, glutathione, catalase, BHT and oxalic acid) the visible browning was reduced and a slight increase in the percentage of explants showing callus formation could be observed (Table 4.1). Explants incubated on media containing caffeic acid or peroxidase reacted more or less similar to the controls. Foetal Calf Serum, salicylic acid and AVG resulted in decreased percentages of callus forming explants and in more severe browning. Addition of jasmonic acid almost completely prevented callus

Table 4.1 Effect of the application of antioxidative substances during incubation of tulip bulb scale explants. Values are mean ± SD.

treatment	% explants forming callus
control	52 ± 18
trolox	73 ± 20
glutathione	68 ± 26
catalase	67 ± 16
BHT	66 ± 11
oxalic acid	61 ± 13
caffeic acid	43 ± 29
peroxidase	39 ± 16
Foetal Calf Serum	30 ± 17
salicylic acid	28 ± 18
AVG	26 ± 16
Jasmonic acid	9 ± 9

formation and stimulated browning.

None of the tested treatments resulted in a changed phospholipid fatty acid composition which differed from the standard incubations; the increase in linolenic

Table 4.2 Schematic overview of differences in stress-related enzymes or TBArs after addition of antioxidative substances during incubation. In this table a ↓ means a decrease when compared to incubation on standard medium and a ↑ an increase. No arrow indicates no clear effect of the treatment.

Reaction of the tissue	treatment	POD	CAT	SOD	TBArs
positive	trolox		↑	↓	↓
	glutathione				↓
	catalase	↓	↓		
	BHT			↓	↓
	oxalic acid	↓			
neutral	caffeic acid	↓			
	peroxidase				
negative	Foetal Calf Serum				
	salicylic acid	↑	↑		
	AVG				
	Jasmonic acid	↓	↓		↑

and decrease in linoleic acid were identical for all tested additions (data not shown).

For some enzymes, changes in the activity pattern could be found (Table 4.2). These changes were not very consistent but a decrease in SOD and in the production of TBArs was only observed with a number of the positive treatments. Low activities for peroxidase and catalase and a higher production of TBArs were observed upon addition of jasmonic acid, a treatment which resulted in a very low number of viable explants. Salicylic acid resulted in higher activities of peroxidase and catalase. Most differences were seen in the first two weeks of incubation.

Submerged cutting of explants:

Explants which were cut submerged, all turned brown after about 4 weeks of incubation. Although submerged cutting seemed favorable in the period directly after cutting with respect to a lowered production of TBArs (see van Rossum and van der Plas 1995), this did not lead to any long-term positive effect on the vitality of the explants (Table 4.3) and little callus formation was observed. Best results were obtained when explants were cut in air as in the standard incubation.

Table 4.3 Reactions of bulb scale explants to different treatments applied either only during submerged cutting (A), during incubation after submerged cutting in medium without further additions (B) or both during submerged cutting and subsequent incubation (C).

treatment	reaction of tissue when compared to control
trolox (A,C)	less browning in first phase.
phenidone (A)	in later stages more browning than in control
detapac (A)	severe browning of the explants
paclobutrazol (B)	severe browning of the explants

No differences in the activities of stress related enzymes could be found when explants, which were cut submerged, were compared with explants cut in air. Cutting of explants submerged in medium containing antioxidative substances (especially trolox) had positive effects (Table 4.4) on the activation of a number of stress-related enzymes. Addition of trolox resulted in a lower lipoxygenase activity both when applied during submerged cutting, and in the incubation medium after submerged cutting; this effect of trolox was seen both on the initial peak in LOX activity and on the overall activity during the incubation period. Application of phenidone during the cutting resulted in a lower activity of LOX during the first hours after the cutting,

Table 4.4 Schematic overview of differences in stress-related enzymes or TBArs after addition of antioxidative substances only during submerged cutting (A), during incubation after submerged cutting in medium without further additions (B) or both during submerged cutting and subsequent incubation (C). In this table a ↓ means a decrease when compared to dry cutting and incubation on standard medium an a ↑ an increase. No arrow indicates no effect of the treatment.

Treatment	phase	LOX	POD	CAT	SOD	TBArs
medium without additions	A					↓
trolox	A	↓	↓		↓	↓
	C	↓	↓		↓	↓
phenidone	A	↓			↓	
detapac	A	↑		↑		
paclobutrazol	B		↑	↑		

afterwards the same activity as in the controls was found. The use of detapac in the cutting medium resulted in higher activities of catalase and LOX after 1 week of incubation, but did not influence the activities of these enzymes in the first days after the cutting of the explants. Paclobutrazol applied in the tissue culture medium after submerged cutting resulted in a higher activity of peroxidase and catalase after 1 week of incubation; the addition of trolox and phenidone did not influence the activity of catalase, phenidone and detapac did not influence peroxidase activity.

Discussion

Upon incubation of tulip bulb scale explants a number of changes in physiological parameters can be observed. However, their role in the unsatisfactory regeneration of shoots on these explants is not always clear. The changes in fatty acid composition seem to be a general reaction of the tissue to its changed environment since it is observed in all experiments we performed (see also chapter 2), and cannot be influenced by changing the conditions during the incubation period. During the adaptation to the changed environment, oxidative stress is occurring, as indicated by the production of TBArs and the activation of stress-related enzymes like peroxidase, catalase and SOD. As regeneration of shoots from bulb scale explants generally is not satisfactory (this in contrast to the well regenerating stalk explants), the question arises whether it is possible to improve the developmental reaction by interfering with this oxidative stress reaction.

However, no significant improvement of the regeneration could be observed in our system by the addition of a wide variety of (anti-oxidative) substances which were reported to have positive effects in tissue culture (Saleem and Cutler 1987; Roustan et al. 1992; Olmos et al. 1994; Chauchan et al. 1992; Kuroda et al. 1991; Housti et al. 1992). In some treatments, an improved antioxidative capacity, as well as a slight inhibition of browning of bulb scale explants could be observed; however no improved shoot formation on the explants was found. This suggests that the tissue has either a sufficient anti-oxidative capacity or that the concentration of the added compounds was not high enough to prevent the deleterious effects of the reactive oxygen species. Since we used concentrations that were effective for other systems, a

lack of effect due to a too low concentration was not expected. The inhibition of browning observed for a number of anti-oxidative substances, suggests a slightly improved antioxidative capacity of the tissue, but simultaneously suggests that regeneration in this system cannot be improved by the presence of an improved oxygen stress defence mechanism.

The use of an ethylene inhibitor (AVG) to reduce ethylene production via the enzymatic route did not improve the regeneration potential of the bulb scale explants, but led to an even more negative performance of the explants. This might indicate that ethylene has no inhibiting effects in our system; possibly it has even a role as a messenger in the wounding reaction and stimulates the wounding response: ethylene can regulate its own biosynthesis (Biddington 1992). This is another indication that oxidative stress, mediated by ethylene does not cause the poor regeneration of tulip bulb scale explants. Submitting the tissue to jasmonic acid in order to stimulate the wounding response (Rakwal 1996; Farmer and Ryan 1994) led to a decreased viability of the tissue and a low activity of the stress-related enzymes. Possibly, the addition of jasmonic acid to the nutrient medium leads to a continuous wounding response resulting in low viability and thereby preventing the start of regeneration.

Submerged cutting was not positive for the vitality of the tissue after an incubation period. This might indicate that a reduced production of TBArs directly after cutting explants (e.g. as result of submerged cutting, van Rossum and van der Plas 1995), has no influence upon vitality and the further developmental reaction of the tissue. On the contrary, even a negative effect could be observed of the submerged cutting, possibly caused by the increase in concentration of the medium components on the surface of the explants during the drying of the explants after submerged cutting; locally very high salt or antioxidant concentrations might occur, leading to damage to the cells. A second possibility might be that substances, produced during the wound reaction which also possess a signal function (e.g. hydrogenperoxide, Foyer et al. 1997) are washed away during this treatment, hence preventing the initiation of the wound healing.

Summarizing, the reaction of tulip bulb scale explants seems to be independent of the presence of antioxidative substances in the tissue culture medium. Also from the inconsistent differences between the activities of the stress-response related enzymes for the different treatments, we concluded that the addition of antioxidative

substances in the concentrations tested does not lead to a positive effect on the regeneration of the bulb scale explants, although the vitality may be improved by these concentrations. This conclusion is supported by previous results, obtained from experiments with altered oxygen conditions (chapter 3). In these experiments the response of the bulb scale explants appeared to be largely independent of high (100%) or low (2%) oxygen conditions during incubation, suggesting that reduction or increase of oxygen stress does not influence the vitality of the bulb scale explants.

All these results suggest that the low viability and lack of regeneration of tulip bulb scale explants is not caused by an insufficient oxygen-stress defence mechanism.

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Chapter 5 :

Tulipaline and tuliposide in cultured explants of tulip bulb scales

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Summary

We developed a method for rapid and reliable determination of tulipaline A and tuliposide A. In this process we identified two isomers of the tuliposide A which are spontaneously interconverted into each other. In bulb scales, relatively large amounts of free tulipaline A were found to be present in the tissue and a low amount of tuliposide A. In young developing shoots, the situation was reversed: tuliposide is the main component but the absolute amounts are much lower. During the tissue culture period an increase was found of both tulipaline A and tuliposide A, especially in bulb scale explants. This increase was temporary and followed by a decrease. The increase in tulipaline A was observed both in regenerating and non-regenerating explants although it seems to be higher in non-regenerating bulbs thereby suggesting a role of tulipaline A in the response of bulb scales to incubation *in vitro*.

Introduction

In plants, a large number of substances are found which possess fungitoxic or bacteriotoxic activity. Among these are the tulipalines and their suggested precursors, the tuliposides, which occur in various members of the Liliiflorae and Alstroemeriaceae (Slob 1973; Slob et al. 1975). The best characterised tulipaline is tulipaline A (α-methylene-γ-butyrolactone, fig. 5.1); tulipaline B (γ-hydroxy-α-methylene-butyrolactone) has also been described but has not been as extensively

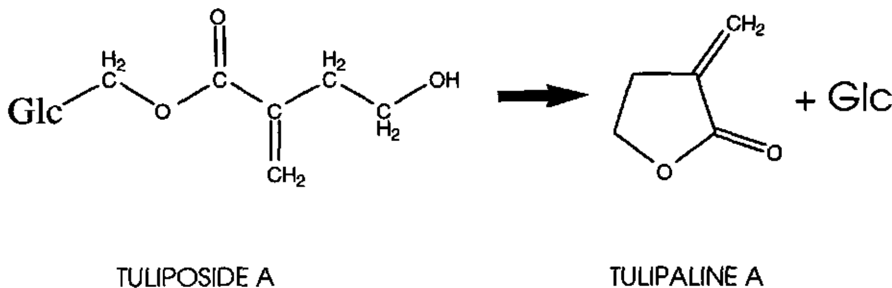

Figure 5.1 Molecular formulas of tulipaline A and its putative precursor, tuliposide A

studied as tulipaline A. Already in 1946 tulipaline A was isolated from *Erythronium americum* (Cavallito and Haskell 1946). In later years tulipaline A was isolated from tulips and identified as a substance with fungitoxic activity. In this study also the precursor of tulipaline A was identified as the glucose ester of α-methylene-γ-hydroxybutyric acid (tuliposide A, Tschesche et al. 1968, 1969). Recently a number of different tuliposides were isolated (Christensen 1995a,b) from *Alstroemeria revoluta* and identified by chromatography and subsequent NMR-analysis. A correlation between the resistance against *Fusarium oxysporum* and tulipaline A content during the annual lifecycle of different tulip varieties was suggested (Bergman 1966). α-Methylene-γ-butyrolactone was also identified as being the causative agent of a contact allergy to flower bulbs ("tulip fingers", Hausen et al. 1983; Gette and James 1990; Hjorth and Wilkinson 1968).

It is difficult to distinguish between the activities of tulipaline (A) and tuliposide (A) because of the unstable nature of the tuliposides. The reported activities (e.g. allergenic reactions) probably are the result of the activity of the aglucon (tulipaline A) which is released from the precursor; the biological activity of the glycoside that is mentioned by a number of authors probably indicates the release of the aglucon. This release of the aglucon can be spontaneous as a result of a pH change (the tuliposide is unstable above pH 5.5) or the result of enzymatic liberation (Verspijck-Mijnssen 1968; Beijersbergen 1969). When the α-methylene-γ-hydroxybutyric acid is released from the glycoside, the lactone is produced by spontaneous lactonisation of the butyric acid at high pH-values (above 5.5) (Beijersbergen 1969; Tschesche et al. 1969). This lactone is very reactive, especially in combination with free radicals; it is described as a copolymerisating substance or it might even polymerise with itself (Akkapeddi 1979; Beijersbergen 1969). This could lead to polymerisation or cross-linking of cell constituents and prevent any biological function of this cell constituent. It has also been described as being reactive in Diels-Alder type reactions and as being sensitive to nucleophilic reagents (Grieco and Mayashita 1975; Danishefsky et al. 1979). This might result in the reaction of the tulipaline with molecules containing double bonds such as unsaturated fatty acids or with molecules containing phenolic rings i.e. aromatic amino acids. Release of the lactone might result from wounding of the tissue in which the glycoside is present e.g. after infection by a fungus. The free, reactive lactone might hamper penetration of the

fungus, either by some direct effect on the pathogen or by causing a necrotic spot which acts as a defensive layer of dead cells, preventing further penetration (Beijersbergen 1972). In accordance with this hypothesis, the highest amount of tulipaline in bulbs is found in the outermost bulb scale, thus providing the best defence against infection by fungi.

Micropropagation of tulip inevitably starts with cutting an explant. As a consequence, tulipaline will be released which might affect the survival of the cells adjacent to the wound surface. A decreased viability of the explants and therefore a decreased regeneration potential due to this tulipaline was suggested in previous research on micropropagation of tulip bulb scale explants (Koster 1993). Therefore we investigated changes in the presence of tuliposides and tulipalines in bulb scale explants during incubation. This was compared with the amounts of tulipaline and tuliposide in stalk explants, a tissue which regenerates very well in tissue culture (LeNard 1980; Rice et al. 1983). A new HPLC method for the determination of tulipaline was developed, allowing the use of small amounts of tissue.

Material and Methods

Bulb material

Tulip bulbs (*Tulipa gesneriana* L.) cv. Apeldoorn were field grown and harvested in early July. After drying they were stored at 30°C for two to four weeks. Immediately after this period explants were cut (end of July / August). For use in the stalk explant experiments, bulbs were stored at 20 °C until the middle of October followed by 17 °C until November. The bulbs were planted in pots with soil and stored at 9 °C for 6 weeks, where rooting and some sprout development occurred. After this treatment the pots were stored at -2 °C prior to use (Gude and Dijkema 1997).
Most experiments were performed with cv. Apeldoorn. For comparison also cv. MleFeber, Gander and Lustige Witwe were tested.

Tissue culture

Tulip bulb scale explants

After removal of the brown skin, bulbs were longitudinally cut in four parts. After rinsing first with 70% ethanol and then with water to remove remaining ethanol

(each 30 seconds), the bulb parts were sterilised in 1% hypochlorite solution for 30 minutes. After sterilising, the bulbs were rinsed three times in sterile water. The second and third outermost scales were used as starting material. The explants were cut from the basal part of the scale, just above the basal plate; in our experiments we used only the lower two explants from each scale, so 16 explants per bulb were cut. Explants were ± 2.5 mm thick and weighed 70-100 mg. Explants were put on the medium with the basal side down and grown on full-strength MS medium (Murashige and Skoog 1962), containing 3% sucrose, 0.5 g/l casein hydrolysate (Gibco), 0.1 g/l myo-inositol, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 1 mg/l 2,4-D, 1.5 mg/l BAP and 6 g/l agar (BBL) (Gude and Dijkema 1997) ; the pH was adjusted to 6.0 prior to autoclaving the medium. The explants were cultured for 10 weeks at 20°C in the dark.

Tulip stalk explants

The stalks were isolated from the bulbs at the end of December, the top (containing parts of the flower) was removed and the stalk was surface sterilised as described for the bulb scale explants. Six explants per stalk were cut around the first node, thickness ± 1-1.5 mm. The explants were incubated for 10 weeks on MS medium as described for bulb scale explants (basal side down).

Sample preparation

Samples were taken at different time intervals during the tissue culture period, frozen in liquid nitrogen and subsequently stored at -80 °C. Before analysis samples were ground in liquid nitrogen and transferred to 0.1 M phosphate buffer pH 5.2 containing 10 % methanol. After 5 minutes of ultrasonic treatment (Branson: Bransonic 220) samples were centrifuged in a microcentrifuge (eppendorf 5415C) for 5 minutes at 14000 rpm. The complete extraction method was performed on ice and the samples were injected immediately after centrifugation in the HPLC.

Tulipaline and tuliposide determination

For determination of tulipaline and tuliposide a HPLC (Chrompack (type Gras)) system was used. Separation was performed on a chrompack rpC18 column (15.0 x 4.0 mm). A gradient was run from 10% methanol/0.01% acetic acid to 70%

methanol/0.01% acetic acid. A standard run was performed in 15 minutes. Tulipaline and tuliposide were detected with an UV-detector (Spectra-physics Spectra 100) at 210 nm, the absorbance maximum for tulipaline; for tuliposide an absorption maximum at 206.5 nm was reported (Beijersbergen 1969). Concentrations were determined by comparing integrated peak areas (integrator: Spectra-physics chromjet) of pure α-methylene-γ-butyrolactone standards (concentration range 5.7 μM-5.7 mM) with the sample peak areas. For the calculation of the amounts of tuliposide a correction factor of 1.66 was used (the extinction at 210 nm of 1 mole of tulipaline corresponds with that of 1.66 mole of tuliposide).

Wounding signal experiments

To determine the relation between the distance to the wound surface and the disappearance of tulipaline after wounding, explants were cut from the bulb scales with surfaces of 1.5x1.5 cm, 1x1 cm, 0.5x0.5 cm and 0.25x0.25 cm, respectively. Since both the adaxial and abaxial epidermis layer remained intact in these experiments, the maximal distance from the wounding surface, decreased from 7.5 to 1.25 mm in this series of explants.

Sugar determination

Sugars were analysed by injection on a Dionex HPLC-system equipped with a CarboPac PA100 (4 x 250 mm) column and a pulsed electrochemical detector. Separation was performed using 0.1 N NaOH as an eluent (Ooms et al. 1994).

Phenolics determination

Explants were ground in liquid nitrogen and subsequently extracted in 80% ethanol. The phenolic content of this extract was determined using *p*-coumaric acid as a standard in an assay using the Folin-Ciocalteus phenol reagent (Hyodo et al. 1978).

Standards

Pure tulipaline A (α-methylene-γ-butyrolactone) was obtained from Sigma. A partially purified tuliposide A solution from Alstroemeria was kindly provided by Dr B.M.B. Hausen, Universität-Krankenhaus, Hamburg, West Germany.

Results :

Identification of tulipaline and tuliposide

When analysing extracts from *T.gesneriana* cv. Apeldoorn one peak in the HPLC chromatogram eluted with a retention time similar to pure α-methylene-γ-butyrolactone (at 5.1 minutes, see fig. 5.2A). Pure α-methylene-γ-butyrolactone, when added to the extract, co-eluted with this peak without changing the peak shape. When the gradient in the HPLC-run was changed, both the extract peak and the peak from pure α-methylene-γ-butyrolactone showed similar changes in retention time. When this putative tulipaline peak was isolated and injected on GC-MS, a peak was found which gave mass fragments which were identified as α-methylene-γ-butyrolactone by the GC-MS library (fig. 5.2). All these results suggested that this peak in the extract was indeed tulipaline A.

Identification of the glycoside (tuliposide A) was more difficult because no pure standard was available. In a sample of partially purified tuliposide A, isolated from Alstroemeria (Hausen et al. 1983) three major peaks were found (see fig. 5.2B) one of which co-eluted with tulipaline A (peak III). The other two peaks, which both had a larger area than that of tulipaline, were found at retention times 2.6 and 3.4 minutes. Both peaks were isolated, and after freeze-drying, they were dissolved in 10 % methanol/0.1 M phosphate buffer and injected again on the column to check purity of the sample. This resulted in the same elution pattern for both samples, with two large peaks at 2.6 and 3.4 minutes and a small tulipaline peak (about 3% of total peak area). To identify the two early peaks, both samples were incubated in 0.1 M KOH because tuliposides are unstable at high pH (Beijersbergen 1969; Tschesche et al. 1969). This resulted in only one peak on HPLC at 5.1 minutes, i.e. in release of α-methylene-γ-butyrolactone from both compounds. Furthermore both samples contained glucose when analysed for sugar composition. The amount of glucose appeared to be equimolar to the amount of α-methylene-γ-butyrolactone in both samples (data not shown). Other sugars were not detected.

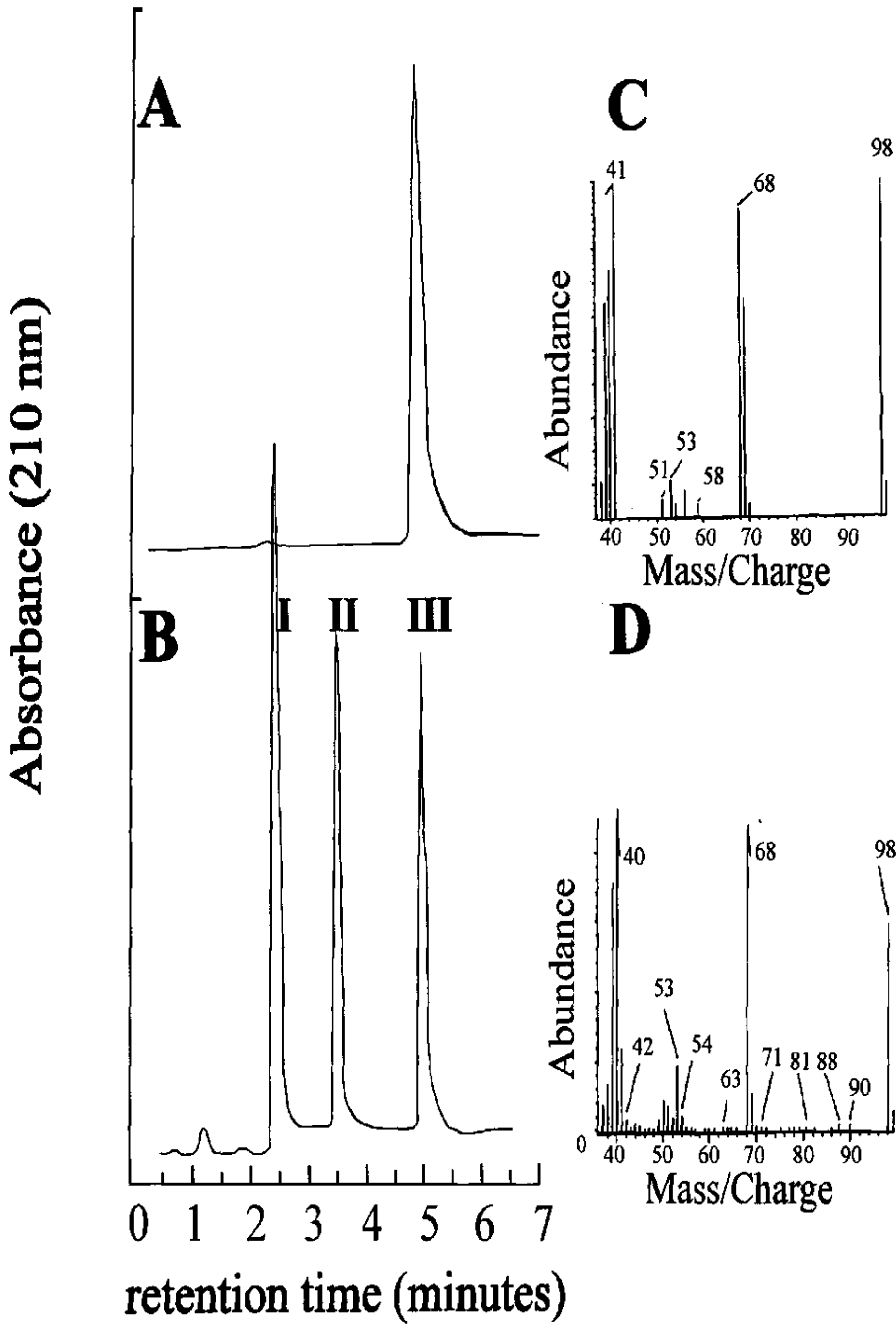

Figure 5.2 HPLC- chromatograms of pure tulipaline A (α-methylene-γ-butyrolactone, Sigma) (A) and of a partially purified tuliposide A extract from Alstroemeria (from Dr. Hausen) (B). Shown are also the mass-spectra as obtained from a GC-MS library (C) or from a GC-MS run of a purified peak III sample of tulip pistils (D).

When this procedure was repeated with extracts from tulip pistils, an organ described to contain up to 30% tuliposide (Tschesche et al. 1968) on a dry weight basis, similar results were found (i.e. the occurrence of three peaks with similar retention times as the peaks found in the partial purified tuliposide sample from Alstroemeria) but the abundance of tulipaline in this sample was lower. Incubation with 0.1 M KOH resulted in the breakdown of the peaks at retention times 2.6 and 3.4 minutes. Extracts made from the same tissue at pH 7.5 contained mainly tulipaline and no detectable amount of the peaks at 2.6 and 3.4 minutes.

Extracts of dry stored bulb material (cv. Apeldoorn) again resulted in these three peaks, but the area of the peaks at retention times 2.6 and 3.4 minutes, was much smaller than that of the tulipaline peak found in these samples. Tulipaline and tuliposide contents of the samples were stable for a least one day when stored on ice; upon replicate injections on HPLC of the samples, no differences both in tulipaline and tuliposide content or in the tulipaline/tuliposide ratio could be observed (data not shown).

Changes in tulipaline and tuliposide contents during incubation of tulip scale and stalk explants

Bulb scale explants of cv. Apeldoorn were incubated for 11 weeks. Samples were taken throughout this incubation period. Two types of samples were analysed: explants from bulbs with a satisfactory reaction in tissue culture (regenerating explants, showing cell division, callus formation and some shoot formation in combination with little browning) and non-regenerating explants (showing no or little callus and shoot formation and more severe browning) as described before (chapter 2). The amount of tulipaline in the explants was in all cases much higher than that of the putative tuliposides (the peaks at 2.6 and 3.4 minutes).

In bulb material, differences in tulipaline content in the different bulb scales were observed. Highest tulipaline concentrations were found in the outermost bulb scale, lower amounts were observed in the second and third bulb scale (from which the explants for incubation in tissue culture were taken; data not shown, see also Koster 1993). Furthermore, large differences in tulipaline content were observed for bulbs of different cultivars (Table 5.1). The highest concentrations were found in cv. Lustige Witwe and Gander, while low contents were detected in cv. MleFeber and

intermediate values in cv. Apeldoorn. The amount of tuliposide was highest in cv. Apeldoorn and lowest in MleFeber; intermediate values were found in cv Lustige Witwe and Gander. (Table 5.1). The tuliposide and tulipaline levels were similar and very low in scales of MleFeber; in Apeldoorn, Gander and Lustige Witwe the tulipaline content of the scales was usually higher than the tuliposide content. Although the absolute amount of tulipaline seemed to vary strongly between individual bulbs, always the same ratio tuliposide/tulipaline was found (results not shown). The degree of visible browning observed in bulb scale explants in tissue culture correlated inversely with these initial tulipaline contents. The level of phenolics formation as determined by the Folin method appeared to correlate reasonably well with the differences in visible browning between the tested cultivars (table 5.1).

Table 5.1 Tulipaline and tuliposide amounts in 2nd and 3rd outermost bulb scales of bulbs of different cultivars at the start of incubation, compared with the phenolic contents after 3 months of incubation of these explants. The level of phenolics at the beginning of the experiments is negligible (less than 0.1 mg/ g fw).

cultivar	Tulipaline (μmol/ g fw)	Tuliposide (μmol/ g fw)	Phenolics (mg/ g fw)
Gander	2.41 ± 0.05	0.21 ± 0.26	4.33 ± 0.92
Lustige Witwe	2.44 ± 0.60	0.18 ± 0.02	4.14 ± 0.83
Apeldoorn	1.35 ± 0.24	0.51 ± 0.20	7.81 ± 0.53
MleFeber	0.03 ± 0.03	0.06 ± 0.03	17.14 ± 3.07

The same pattern of changes in tulipaline and tuliposide content always could be observed for the explants during the tissue culture period (fig. 5.3A). Upon cutting the explants, a decrease in the tulipaline content was observed within the first hour after cutting (fig 5.4). The amount of tulipaline found in the bulb scale explants increased in the next 10-14 days; much higher levels than in the starting material could be reached (fig. 5.3). After this increase the tulipaline content decreased again. The amount of tuliposide also increased during incubation of the explants, although this increase was smaller than for tulipaline. An increase in tulipaline content during incubation was seen both in non-regenerating and in fairly well regenerating bulb scale explants (fig. 5.3A).

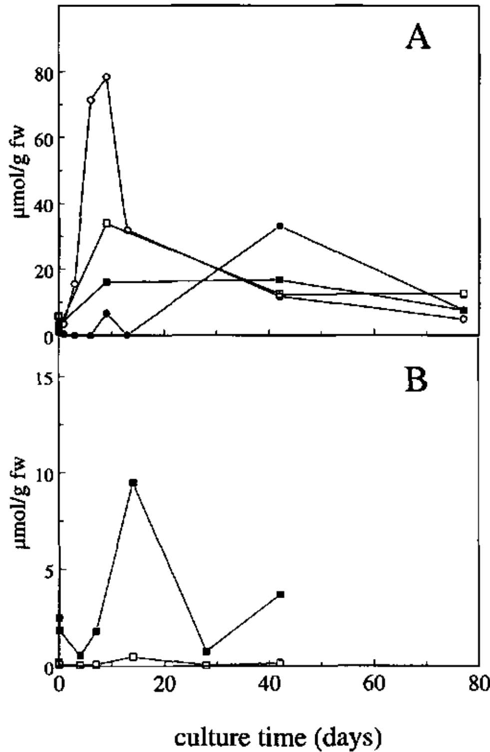

Figure 5.3 Changes in tulipaline and tuliposide content in bulb scale explants (A) and stalk explants (B) of tulip during incubation. Closed symbols represent tuliposide and open symbols tulipaline. For bulb scale explants the changes are shown for regenerating (□,■) and for non-regenerating explants (O,●). The figure gives a representative picture of the pattern of changes occurring in the explants; the overall levels of tulipaline and tuliposide varied strongly between individual bulbs.

The disappearance of tulipaline does not seem to be limited to the cells directly adjacent to the wound surface: although the decrease in the first 15 minutes was especially prominent in small explants (fig. 5.4), also in fairly large explants (15x15 mm) half of the tulipaline disappeared within half an hour after cutting. One to four hours after cutting, all the tulipaline seems to have disappereared from explants up to 10x10 mm. Only in fairly large explants (15x15 mm, fig. 5.4), the distance to the wound surface of the cells in the centre of the explant was large enough to prevent degradation of tulipaline.

In explants from young developing stalks both the amount of tulipaline and of the putative tuliposide, were much lower than in bulb scale explants. In contrast with

the situation in bulb scale explants, tuliposide was the main compound in the tulip stalk explants (fig. 5.3B). Both substances showed a transient increase during the tissue culture incubation; especially the increase and subsequent decrease in both tuliposide isomers were very prominent.

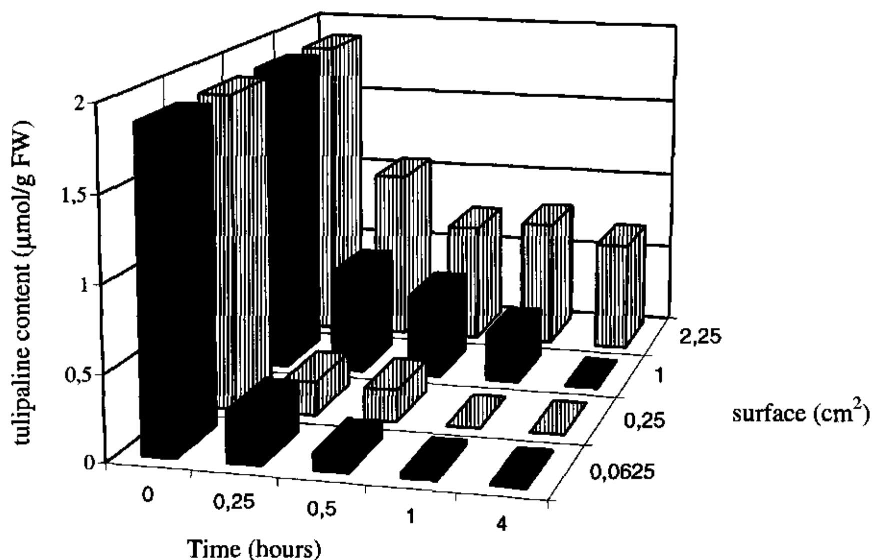

Figure 5.4 Decrease in tulipaline in bulb scale explants related to time of cutting and to the size of the explant.

Discussion

Identification of tulipaline and tuliposide:

From the three major peaks with an absorption at 210 nm found in HPLC-chromatograms of the tested tulip tissues, peak III could be assigned to tulipaline A by comparison with the pure standard at various elution programs.

Peak I and peak II were degraded by alkaline treatments giving rise to tulipaline A. Both compounds also yielded glucose in a 1:1 ratio with tulipaline A. These observations suggest that both peaks represent a glycoside of α-methylene-γ-butyrolactone or of α-methylene-γ-hydroxy-butyric acid which is rapidly converted into the lactone upon hydrolysis. Although a glycosylated form of the lactone was

suggested in the earliest research on tulipaline/tuliposide (Cavallito and Haskell 1946), studies on this subject have proved this to be very unlikely (Tschesche et al. 1968, 1969). Since no other sugar nor any other lactone was found upon alkaline hydrolysis, it is most likely that both peaks are conjugates of glucose and α-methylene-γ-hydroxy-butyric acid (tuliposide A), and not other tuliposides as described by other authors (Tschesche et al. 1968; Christensen 1995a,b). The occurrence of two peaks suggests that two isomers of the tuliposide A are present which are in (chemical) equilibrium. This corresponds with the observation that upon isolation of one peak, re-elution of the isolate results in the re-emergence of both putative tuliposide A peaks in a HPLC-chromatogram at exactly the same position as the original peaks. One possibility is that these peaks represent the 1-acyl-glycoside and the 6-acyl-glycoside of tuliposide A as already suggested by Tschesche et al. (1969). The two forms are said to be easily interconverted by acyl-migration of the α-methylene-γ-hydroxybutyric acid on the glucose molecule (Tschesche et al. 1969).

Tulipaline and tuliposide contents during incubation of tulip scale and stalk explants

Surprisingly, our results show that tulipaline A is the main component in the bulb scales and not tuliposide A, this in contrast to Beijersbergen (1972), who made probable that tulipaline was present in tulip bulb tissue in a bound form as tuliposide A. During our experiments the extraction is performed under conditions in which the tuliposide should be stable (Beijersbergen 1969), therefore a precocious liberation of tulipaline A during the extraction and determination procedure does not seem very likely; this is supported by our observation that the tuliposide and tulipaline contents and the ratio tulipaline/tuliposide in the extracts did not change after being kept on ice for 1 day. Although Beijersbergen (1972) suggested the presence of enzymes, responsible for degradation of the tuliposide to tulipaline and glucose, the conditions of the extraction are supposed to prevent a rapid enzymatic turnover. Indeed, a combined extraction of tulip pistil material (with a high tuliposide content) and bulb scale material did not lead to a significant extra tuliposide degradation; the amounts of tuliposide and tulipaline observed under those conditions were equal to the summed amount found with the separate determinations (98 ± 0.05 % of expected amount of tuliposide). Beijersbergen used mostly outer scales in his experiments, while for

regeneration inner scales were used, which are not rich in tulipaline/tuliposide. However, also in outer scales tulipaline seemed to prevail. The lack of stability of tuliposide at pH-values $\geq$ 5.2 (Beijersbergen 1969) in combination with the pH found in cellular compartments (about 5.5 and 7.5 for vacuole and for cytoplasm, respectively, Kime et al. 1982; Gout et al. 1992) does not support the probability of tuliposide as the main compound.

It doesn't seems very likely that an allegedly reactive substance like tulipaline occurs freely in the cytoplasm of a living cell, since reactions of free tulipaline A with proteins and unsaturated fatty acids are likely to occur (Cavallito and Haskell 1946; Danishefsky et al. 1979), leading to rapid disappearance of the free tulipaline A and to severe hindrance of the biological functioning of the cells. We suggest that tulipaline A might be located in a separate compartment e.g. a vesicle which might provide the means for more or less permanent storage of such a reactive compound. The pH in this compartment should not exceed 6.5, as at high pH values the lactone gradually is converted into α-methylene-γ-hydroxybutyric acid (Beijersbergen 1969) indicating storage in vacuoles or comparable vesicles (Kime et al. 1982, Gout et al. 1992). This does not exclude a role for tuliposide as a precursor of tulipaline; it might e.g. function as a temporary transport form in the tulip. After production in the shoot (where high amounts accumulate in the pistils, Tschesche 1968) tuliposide might also be transported to the bulb scale tissue where a rapid turnover of both forms of this precursor into tulipaline and a subsequent storage in such putative vesicles might occur; as a result no accumulation of tuliposide will be observed. When the tissue is wounded, e.g. during cutting of explants for tissue culture, the tulipaline content rapidly decreases. It might be released and subsequently react with various cell components as described above, eventually leading to cell death and tissue browning. This disappearance (as shown in fig. 5.4) does not seem to be limited to the wounded cells of the tissue: even in a scale explant of 1 cm^2 with only a relatively small wounding surface and a fairly limited number of wounded cells (the mean cell size of bulb scales generally does not exceed 0.1 mm, Koster 1993), no tulipaline can be demonstrated one hour after cutting. Only larger explants (1.5x1.5 cm^2: fig. 5.4) seem to retain tulipaline, suggesting that a considerable distance of cells to the wounding surface is needed to prevent disappearance of the tulipaline. This suggests also the existence of a signal which is able to induce the release of tulipaline from the putative

vesicles in non-wounded cells. This corresponds with the observation of Bergman (1966) that after infection brown spots occur (diameter of 7 mm or more after 8 days of superficial infection), suggesting rapid tulipaline release in cells adjacent to the place of infection inducing cell death in this area by the reaction of tulipaline with cell components and thus inhibiting further extension of the infection by encapsulation of the infection in a layer of dead cells.

An inverse relation was found between the degree of browning (e.g. appearing from the formation of phenolics) of bulb scale explants and the initial amount of tulipaline in the bulbs (Table 5.1). Based on the supposed role of tulipaline in the formation of necrotic spots upon infection by fungi, we expected severe browning in the high tulipaline containing bulbs and not in the low tulipaline cultivars. Apparently, the browning degree is not only related to the initial amount of tulipaline in the scale tissue. This corresponds to our observation that addition of exogenous tulipaline in the medium of bulb scale explants did not induce extra browning but even sometimes prevented it, although it did not improve the vitality of the explants (results not shown). In this respect, one has to take into account that an inhibitory effect of tulipaline on enzyme activities might have two contrasting effects: a faster browning reaction when the interference with cellular integrity leads to cellular decompartmentalisation resulting in the formation of the brown compounds but also to a slower browning reaction when the enzymes involved in the production of these compounds are rapidly inhibited by tulipaline.

Measurements on changes in tulipaline contents in scale explants during incubation showed a considerable potential for tulipaline synthesis during explant incubation, leading to tulipaline levels even higher than in the starting material. These (temporarily) high levels might contribute to the gradual browning and loss of viability of the explants but are probably not the sole reason for these processes since such an increase is also seen in vital explants, although to a somewhat lesser degree (fig. 5.3).

In well regenerating stalk explants the amounts of tulipaline and tuliposide were relatively small when compared to the amounts found in bulb scales corresponding to a potential negative role of tulipaline in shoot development. Especially, the content of tulipaline (the allegedly damaging compound) is very low; in these stalk explants, glucose bound tulipaline (tuliposide) is the main storage form.

This situation is also found in pistils which contain large amounts of tuliposide (up to 30% of dry weight, Tschesche 1968) and low amounts of tulipaline. In stalk explants, no rapid change in the already low tulipaline or tuliposide content upon wounding was observed but during incubation of stalk tissue explants, a small increase in tulipaline and a far more distinctive increase in tuliposide were found. These increases do not seem to hamper the regeneration of the explants. However, the levels found in tulip stalk explants remain low, when compared to those of bulb scale explants.

Summarising, although our results indicate a negative correlation between tulipaline and regeneration/ tissue vitality, no direct negative effect of tulipaline in tulip tissue culture can be demonstrated in contrary to the suggestion made by Koster (1993). No prediction can be made on the vitality and developmental reaction of bulb scale explants based on the tulipaline/tuliposide contents of the starting material.

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Chapter 6

General discussion

Oxygen stress-related problems in micropropagation of tulip

In order to resolve the physiological basis for the problems in tulip bulb scale micropropagation, a tissue that is very recalcitrant in tissue culture as described previously (Koster 1993), we evaluated the role of oxidative damage. Oxygen is a necessary prerequisite for most lifeforms on earth. However, oxygen can be toxic as well (Raven et al. 1994; Scandalios 1993; Pell et al. 1997). The occurrence of oxygen stress in tissue culture has been widely reputed for causing a broad range of problems during regeneration processes or during establishment and growth of plant tissue culture systems (Benson et al. 1992b; Creemers-Molenaar et al. 1992a; Dalton and Morris 1993). Processes related to oxygen stress are studied by numerous researchers (Ishii 1988; Kuroda et al. 1991; Benson et al. 1992b; Benson and Roubelakis-Angelakis 1992; Buonaurio and Kumar 1995; Olmos et al. 1994; Okane et al. 1996) and the activation of oxygen stress response mechanisms are well documented. Oxygen stress-related damage can occur by direct action of free radicals on the membranes via lipid peroxidation resulting in breakdown of lipids (Benson 1990; Benson et al. 1992a), or by secondary effects resulting from free radical damage such as the production of toxic substances, e.g. MDA or phenolics (Benson 1990). This might lead to the assumption that severe browning of tissue culture material is caused by such oxidative stress-related processes (Housti et al. 1992). A generally accepted procedure to improve performance of recalcitrant plant species in tissue culture-mediated regeneration is the use of a variety of additives to the nutrient medium which have proven to be successful in other tissue culture systems, preferably of related species. In a number of cases these additives have an antioxidative function (Saleem and Cutler 1987; Creemers-Molenaar et al. 1992a; Dalton and Morris 1993; Krens and al. 1994). The type and concentration of the antioxidative substances are usually determined by application of substances reported to be successful in other tissue culture systems. However, considerable differences can be observed between different species and even between different cultivars within one plant species. Reports related to direct physiological research on the basis of the oxygen stress-related problems and the mechanism by which the antioxidative substances exert their beneficial effect on performance in tissue culture are more scarce in literature (Benson et al. 1992a; Okane et al. 1996; Piqueras et al. 1996). Attention has focused primarily on the activation of stress-related enzymes (e.g. peroxidases, catalase and SOD), which are either involved

in the protection of the tissue or in removal of the damaging compounds produced during wounding and incubation. In the second place, components that are produced as a result of oxidative damage (e.g. ethylene or TBArs; Housti et al. 1992; Cherif et al. 1996) are measured. Interpretation of the results is often difficult: if a tissue shows a strong activation of stress response-related enzymes, this might mean that the tissue is suffering from severe stress, but it might also indicate that the tissue is very well capable to process the reactive oxidative species, thereby preventing any damaging events. This is e.g. shown by the high SOD acitvities found in stress-tolerant or stress-adapted plants enabling them to live under stress conditions (Benes et al. 1995, Piqueras et al. 1996).

In our project, different approaches were used to evaluate the role of oxygen stress: the oxidative stress was modulated by application of different oxygen concentrations in the headspace of the tissue culture vessel, or the antioxidative status was influenced by the addition of antioxidants to the nutrient medium. Changes in the enzymatic response system as well as in the membrane composition and in TBArs content were measured. The significance of the observed changes was evaluated by comparing explants which showed different reactions in tissue culture: explants from the same plant organ (scales tested in different seasons resulting in different regenerative potentials; Koster 1993), well regenerating explants from the same plant species (stalk explants; LeNard 1989), other related, regenerative tissue culture systems (lily scales, Djilianov et al. 1994) and a not-related, well-characterized model system (apple root slices, De Klerk et al. 1995). Our goal was to determine the role of oxidative stress phenomena in the poor regeneration and low vitality of tulip bulb scale explants: our working hypothesis was an extreme sensitivity of tulip bulb scale explants to oxidative stress-related processes.

However, we did not find a clear proof to confirm the hypothesis of extreme sensitivity of tulip bulb scale explants to oxidative stress. The activation pattern of stress-related enzymes (SOD, LOX and PAL) in the period directly after cutting the explant, when most damaging events are taking place, was not principally different in both regenerating and non-regenerating bulb scale explants and, moreover, in well regenerating tulip stalk explants (chapter 2). A relation between high activities of oxygen stress-related enzymes (LOX, catalase, peroxidase and PAL) and low viability of the tissue was found only in later stages of incubation. This is comparable to other

studies (Kuroda et al. 1991; Benson et al 1992a; Benson and Roubelakis-Angelakis 1992; Olmos et al. 1994; Piqueras et al 1996) where higher activities of the stress-related enzymes are found in cultures with declining viability. A further comparison of the activities of the oxygen stress related enzymes found in these studies with the activities found in our studies show that the activities observed for tulip tissue were not extremely high or low. This suggests that high activities of oxygen stress-related enzymes do not cause low viability but rather are the consequence of loss of viability by the tissue. A continuous stress to the tissue is the result of this gradual loss of viability, leading to induction of defence mechanisms, indicated by the high activity of stress-related enzymes. This is in contrast with the situation in vital tissues (i.e. regenerating scale explants or stalk explants (chapter 2): after processing the initial amount of free radicals generated by a "stressing" event (e.g. cutting an explant) the activity of stress-related enzymes decreases, again indicating a low level of stress experienced by the explants in this situation. Since the level of most oxidative stress related enzymes in regenerating explants reaches the same values as in non-regenerating explants during the first period after cutting, it is likely that both have experienced the same amount of stress which is overcome by the regenerating explants but not by the non-regenerating explants as shown by the continuous high activity of stress related enzymes in the latter.

A relation between tissue vitality and changes in fatty acid composition of membrane phospholipids could not be found in our experiments, although a increase in saturation of membrane phospholipids is to be expected in case of oxygen stress, caused by the relatively higher sensitivity of double bonds for oxidative attack (Ohlroggė and Kernan 1982; Gardner 1989). However, in our experiments we even found a higher double bond index in incubated explants when compared to the starting material, because the amount of phospholipids containing linolenic acid (C18:3) increased and linoleic acid (C18:2) showed a corresponding decrease. These changes in composition were found both in regenerating and non-regenerating scale explants and were not related to tissue culture performance of the explants. We concluded that the changes in fatty acid composition were the result of adaptation of the tissue to its new physiological programming, because the resulting fatty acid composition closely resembles that found in stalk material (cut from growing tulip tissue, chapter 2). No

indications were found that the changed fatty acid composition of the tissue might be connected with severe oxidative damage of the bulb scale explants.

Moreover, in experiments aimed at decreasing the oxygen stress by incubation under low oxygen (chapter 3), we did not observe an improved regeneration of shoots on the explants. Low oxygen conditions in order to reduce oxygen stress, always restrained the various regenerative responses (chapter 3). Possibly the evolution of reactive oxygen species may function as a signal for initiation of regeneration directly after cutting explants (DeMarco and Roubelakis-Angelakis 1996; Burdon 1996, Suzuki et al. 1997). Of course, a too low availability of oxygen also might limit respiration and energy production, in that way also preventing a proper and fast regeneration reaction. This dual role of high oxygen as both source of reactive oxygen species and prerequisite for growth might mean that if tissues are capable to process the increased amount of reactive oxygen species during the high oxygen conditions, the higher concentration of (non-reactive) oxygen is beneficial for respiration and growth and reactive oxygen species are not present or necessary to provide a continuous signal to maintain the growth.

Also in transfer experiments in which after an initial low oxygen treatment, the explants were placed under atmospheric oxygen conditions, no satisfactory regeneration was observed. On the contrary, the use of high (100%) oxygen conditions, supposed to be detrimental to the tissue (Quebedeaux and Hardy 1975; Trippi et al. 1988), resulted in an even better outgrowth of the regenerating structures on stalk explants after their formation. Although in the dedifferentiation and initiation stage of new primordia, high oxygen conditions are restraining, the absence of harmful effects of high O_2 was confirmed by experiments in which we evaluated the response of other tissues (apple stem slices and lily scales) to high oxygen conditions. These results are contradictory to those of other authors and might implicate that not the high oxygen conditions but the physiological responses differ for the tissues: the activation of e.g. stress-related enzymes like SOD and catalase is not always related to high oxygen (Foster and Hess 1980). This suggests that in order to cope with high oxygen and/or reactive oxygen species, other physiological factors next to the enzymatic defence systems are important.

In the experiments in which we added antioxidative substances (chapter 4), only small changes in the oxidative stress-response but no clear positive effects of

these additions were observed. The response of the tissue with regard to the activation of the oxygen stress response related enzymes, changes in phospholipid composition and the production of phenolics and TBArs were more of less identical in all experiments we performed. Only a temporary small reduction of the amount of TBArs produced in the first hours after cutting the explants could be observed, if the cutting of the explant was carried out under reduced oxygen availability (submerged cutting; van Rossum and van der Plas 1995). However, none of the tested antioxidative substances resulted in a clearly promoted shoot regeneration as was expected, although for a number of additions a slight visible improvement of callus formation on the explants was seen (table 4.1). Since the tested concentrations were successful in literature and small effects (less browning) were seen an improved antioxidative status was suggested. Our conclusion from these experiments was that this improvement of the anti-oxidative status of the tissue generally is not sufficient to improve viability and subsequent regeneration of shoots on explants of tissue culture systems suffering from low viability under standard conditions, suggesting that the antioxidative status of the explants is sufficient and not the limiting factor.

Our results do not point to oxidative stress as being the primary cause for the unsatisfactory regeneration in tulip bulb scale explants. The browning of the tissue probably is just a secondary effect of the low viability of tissue that is not able to survive the process of cutting explants and to regenerate new, viable shoots. This is comparable with the observation of Buonaurio and Kumar (1995) who found only high activities of lipoxygenase, SOD, peroxidase and catalase about 2 weeks after infection with Xanthomonas in pepper leaves when the vitality of the tissue had decreased as result of the infection. Since the physiological reaction of the explants is similar in regenerating and non-regenerating explants and the activity patterns of oxygen stress-related enzymes are generally comparable to the patterns observed in well regenerating tulip stalk explants, this is considered a further indication that the browning in the non-regenerating explants is a secondary effect at most. Our results suggest that the antioxidative response of the tissue is sufficient and other physiological factors might play a more important role.

Tulipaline A in tissue culture

In our second hypothesis we suggested a role for the unsaturated lactone α-methylene-γ-butyrolactone, also known as tulipaline A, in the poor regeneration of tulip bulb scale explants. This substance is thought to be involved in an anti-fungal defence mechanism of tulip bulb scales and supposedly very reactive. Harmful effects therefore are to be expected, especially as a consequence of cutting and incubating of bulb scale explants (Bergman 1966; Tschesche et al. 1968; Beijersbergen 1969; Beijersbergen and Lemmers 1975; Koster 1993). Similarities between the brown spotting of tulip bulb scale tissue upon fungal infection and browning in tissue culture led us to the hypothesis that tulipaline might be the common cause of these browning processes: the stress involved in cutting and incubating is similar to the stress caused by the wounding of tissue after fungal infection.

After development of a HPLC-based method for the determination of small amounts of tulipaline A and tuliposide A in tulip material, we measured the concentrations of both substances in different tissues. To our surprise the supposedly very reactive compound tulipaline A appeared to be more abundant in the bulb scale tissue than its putative precursor/storage form, tuliposide A. Various control experiments proved that this is not the result of a precocious liberation of tulipaline A from tuliposide A (chapter 5). In stalk tissues we found the reversed situation (tuliposide A was here the main component), although the concentration of both substances was lower than in bulb scales. High concentrations of tuliposide A were found in tulip pistils in similar amounts as described by Tschesche (1968), indicating that our isolation and determination methods were valid and did not lead to an undesired conversion of tuliposide A into tulipaline A (see also chapter 5). Our observations do not correspond with those of Beijersbergen (1969) who concluded that tulipaline A in the scale tissue was present in its glycosylated form, tuliposide A.

The intracellular pH value found in most plant tissues is a point in favour of tulipaline A being present instead of tuliposide A. Because the cytoplasm generally has a pH of about 7.5 and the vacuole a pH of about 5.5 (range 5.0-5.8 in various plants, Kime et al. 1982; Gout et al. 1992), both compartments form an environment in which tuliposide (unstable at pH-values above 5.2, Beijersbergen 1969) would be converted into tulipaline. After prolonged incubation of tulipaline A at pH-values above 6.5 the molecule is converted into the butyric acid form (Beijersbergen 1969),

so storage in the cytoplasm of the lactone form is not possible for long periods. The tulipaline is expected to be present in an environment where the reactive molecule does not cause any problems to the cell constituents. Also for that reason, we do not expect it to occur freely in the cytoplasm, however it might be present in the vacuole or a specialized cell compartment.

During tissue culture of bulb scales we found a decrease in the amount of tulipaline A directly upon cutting the explant, indicating a reaction of the tulipaline in the tissue, e.g. with DNA or proteins. The extent of this decrease seemed to be related to the ratio of the wound surface to the total size of the explants, but even fairly large explants seem to lose all their tulipaline. This indicates that tulipaline is also released from the putative vesicles in non-wounded cells, suggesting the existence of a signal traveling through the explant. This release is followed by production of tulipaline A in the weeks after cutting. To evaluate the significance of the tulipaline A content in the tissue culture response of tulip bulb scale explants, regenerating and non-regenerating bulb scale explants were compared. Both regenerating and non-regenerating explants showed an increase in the amount of tulipaline A upon incubation, although non-regenerating explants produced more tulipaline A in the first weeks of incubation. Probably the high level in non-regenerating explants was related to the high stress that this tissue experienced and its low viability. However, directly after cutting no differences between regenerating and non-regenerating bulb scale explants were found. As tulipaline is produced both in regenerating explants and during the incubation of non-regenerating explants with low viability, we concluded that in fully functional tissue the tulipaline content might be fairly high and does not seem to be directly related to low viability. In addition, when cultivars differing in tulipaline contents were compared, high initial tulipaline contents seem to result in little browning in tissue culture (chapter 5). All these results do not confirm our hypothesis that micropropagation starting from tulip bulb scale explants does suffer specifically from tulipaline present in or produced by the explants. The amounts of tulipaline are probably not high enough to be really detrimental to the tissue and the reactivity of tulipaline A is not as high as previously supposed.

This reactivity of tulipaline is also described in dermatological literature as the cause of a contact allergy. In this literature, a proposed remedy is the use of cysteine containing lotion (Gette and James 1990). Tulipaline is rendered harmless by

nucleophilic addition (of the Michael type) in which the tulipaline A functions as the Michael acceptor (Grieco and Miyashita 1975) and cysteine as the donor. This implicates that reactions of tulipaline might occur with cysteine residues of (enzymatic) proteins in the cytoplasm, in that way hampering the biological functions of these enzymes. However, in experiments in which we tested the influence of tulipaline on *in-vitro* enzyme activity (e.g. 6-phosphogluconate dehydrogenase (6PGDH), PAL or PPO) no large influence could be found (unpublished data), suggesting that the reactivity via this mechanism is not very high. The reactivity of tulipaline can also be caused by Diels-Alder type reactions in which the double bonds of the methylene group and the aldehyde group react with other double bonded molecules (Danishefsky et al. 1979). A third possibility for a reaction mechanism is the activation of tulipaline A by a free radical mechanism, analogous to the activation of this molecule in polymer chemistry (Akkapeddi 1979; Stansbury and Antonucci 1992). The reaction conditions of these experiments are not described very exactly in this literature. Akkapeddi only gives the temperature of 60°C at which he incubated the mix of organic compounds, tulipaline A and a free radical producing agent. Stansbury and Antonucci (1992) used tulipaline A in dental resins, implying not very stringent reaction conditions.

In the literature a number of possible effects of tulipaline in different systems are described although the effects are sometimes contradictory. Some authors describe a negative effect on bacterial swarming or on human lung carcinoma cell lines (Woerdenbag et al. 1986; Lenz and Süssmuth 1987) while other authors have found positive effects like antimutagenic, antiinflammatory or cytoprotective activities (Kuroda et al. 1986; Prestera et al. 1993; Maria et al. 1995; Hayashi et al. 1996). The beneficial or harmful effects of tulipaline may be dose dependent; Diamond et al. (1986) describe that tulipaline can slightly enhance mutation-rates at low concentrations, whereas concentrations higher than 50 μg/ml significantly depress mutation rate in *E. coli* bacteria. Lenz and Süssmuth (1987) describe an induction of swarming of *Proteus mirabilis* at low tulipaline concentrations and an inhibition by high concentrations.

The conditions in plant cells (neutral or slightly acid pH) will not cause the aldehyde group to become a strong nucleophilic compound by protonization, so nucleophilic addition will not occur very easily. In connection with a free radical

reaction mechanism, the role of tulipaline A in the fungitoxic response in plant materials might also be related to the presence of reactive oxygen species; the reactivity then strongly increases under the influence of the free radicals produced in the wounding-response. Indeed, we observed an interference of tulipaline in enzyme assays in which reactive oxygen species play a role, especially in the SOD assay in which oxygen radicals are produced (unpublished data). Because of its sensitivity to radicals, pure tulipaline A is stabilised by addition of 2 % BHT (Sigma 1997), a strong antioxidant preventing polymerization (Flores et al. 1994). A relation between tulipaline and reactive oxygen species is suggested by Hayashi et al. (1996) who describes the activation of NAD(P)H:(quinone acceptor) oxidoreductase (a free radical protection mechanism) in *E. coli* by tulipaline.

We therefore suggest that reactivity of tulipaline in a wounding response is increased by interaction with reactive oxygen species. These reactive oxygen species might also act as the "signal" we proposed in chapter 5 for the release of tulipaline in reaction to wounding of the tissue. Probably the tulipaline is stored in the vacuole, there being not extremely reactive; after wounding the tulipaline is activated by free radicals causing increase of its reactivity, leading to several harmful reactions with cell components and finally death of the cells adjacent to the wounded surface. This process can be evaded if the wounding/ healing response is strong enough to remove the reactive oxygen species. As we showed above, this is probably the case in tulip bulb scale explants. As a consequence no significant negative influence of tulipaline A in tissue culture performance is to expected.

Conclusions

From our results based on the experiments in which we evaluated the role of oxidative stress and oxidative damage in micropropagation of tulip bulb scale explants, no apparent role of these phenomena in the poor viability and unsatisfactory regeneration of these explants can be concluded. In all our experiments, no significant differences in oxidative stress-related parameters between viable, regenerating and low-viable, non-regenerating explants at the start of incubation could be found. Differences were found only in later stages of incubation and therefore were probably the result of low vitality of the tissue. Production of oxidative damage-related substances did not differ between these two groups. Also from the changes in the

membranes no differences could be concluded. Furthermore, the fact that vitality and/or regeneration could not be influenced by antioxidative treatments or changed oxygen levels further supports this conclusion.

Also no clear evidence for a role of either tulipaline A or its putative precursor in the unsatisfactory regeneration response could be established. Although some changes in tulipaline content during incubation on nutrient media were found, no correlation with vitality of explants of these changes could be established. Since a rapid change upon cutting could be found, we suggest that there is a relation between tulipaline A and the wounding response. Possibly the tulipaline A is activated by free radicals generated as a result of wounding.

Since the tested hypotheses did not lead to an answer on the cause of low viability and unsatisfactory regeneration of tulip bulb scale explants, this problem has probably another origin. Since the regenerability decreases in time after the harvest of the bulbs, it may be related to the developmental processes going on in the tissue. From the work of Koster (1993) an important role of the developmental stage of the tissue used for the regeneration of shoots can be concluded. Explants from prematurely lifted bulbs, which did not get a high-temperature treatment (30°C), did not show the initiation of shoot regeneration. Such high temperatures are described to be necessary in tulip tissue for continued differentiation (LeNard and DeHertogh 1985). However, this high temperature, needed to enable regeneration, might also lead to an accelerated development of senescence related processes. Possibly this gradual progress of senescence after harvest finally reaches a stage where no further dedifferentiation and regeneration can be accomplished. This might e.g. be caused by loss of genetic material which has been described to occur in specialised, older plant tissue (Wernicke and Bretell 1980). A relation between loss of regeneration potential and loss of genomic material in *Lolium* suspension cultures has been reported previously (Creemers-Molenaar et al. 1992b). It is recommended to test this hypothesis, and subsequently develop a method, which enables the arrest of bulb material in a physiological stage which enables it to regenerate shoots on viable explants. This has been proven possible for stalk material by the use of "ice tulips": it may also be possible to design a method for the use of bulb material with a satisfying regeneration potential throughout the year, instead of only in July/August.

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Summary

The propagation of tulip is severely hampered by the slow traditional propagation methods. With the increased need for new varieties with e.g. an improved resistance against pests, next to the continuous interest in new varieties with e.g different colors, the 25-30 years needed before a new cultivar can be commercially introduced is a problem. Therefore, alternative, faster methods to propagate tulip are wanted and the application of tissue culture techniques seems very promising. However, still a number of problems exist; especially tissue culture starting from tulip bulb scales has been proven cumbersome, because the explants are only vital and able to regenerate shoots (from which new bulbs can be produced) in a very limited period of the season. Our project was aimed at the elucidation of the physiological factors responsible for this loss of vitality and regeneration potential.

Two hypotheses were tested. In one hypothesis the role of oxidative stress in these problems was evaluated. If tissues are not able to process the reactive oxygen species produced during the cutting of an explant or to cope with the stress during the incubation, the tissue can be severely damaged by oxidative stress phenomena like membrane damage, production of toxic substances (TBArs or phenolics) and damage to DNA or structural proteins, leading to loss of viability and death of the explants. In our second hypothesis the role of tulipaline A (α-methylene-γ-butyrolactone) in the loss of vitality was evaluated: since tulipaline A has been implicated in fungal defence of tulip bulb scale tissue as being a toxic substance to the fungus which is released upon wounding, we hypothesized that release or production of tulipaline A might cause toxic reactions in tulip tissue, leading to loss of vitality and death of the explants upon cutting.

To test our hypothesis of extreme sensitivity to oxidative stress we used different approaches in the first place: we compared physiological parameters of the stress response in regenerating tulip explants (bulb scales and stalk explants) with non-regenerating tulip bulb scale explants. In a second approach we applied different tissue culture conditions (differing oxygen concentrations and anti-oxidative additives to the nutrient medium) and measured physiological parameters implicated in the oxygen stress response (i.e. changes in enzyme activities (LOX, SOD, peroxidase,

catalase, PAL and polyphenoloxidase), membrane composition and production of TBArs and phenolics). To obtain an indication of the role of the changed oxygen conditions a further comparison was made with other tissue culture systems like lily and apple stem segments. This also enabled us to determine in which phase of the regeneration process the changed conditions have the most effect.

The physiological response of regenerating and non-regenerating scale explants did not differ significantly. The response was furthermore comparable to the pattern of changes observed in well regenerating tulip stalk explants. Some differences were observed after 2-3 weeks of incubation; these differences are probably caused by the poor viability of non-regenerating explants and not the cause of this poor viability since most damaging events and the start of regeneration are supposed to occur before the stage in which the differences are found. The experiments with modified oxygen conditions in which we applied 100 % oxygen to increase or 2 % oxygen to reduce oxygen stress also yielded no indications of an extreme sensitivity of tulip bulb scale tissue to oxidative stress. Regeneration was not improved by the application of low oxygen conditions and vitality was not negatively influenced by high oxygen conditions; high oxygen conditions seemed favourable for faster growth of the regenerating structures but slightly negative to the formation of primordia. No improvement of vitality could be observed after addition of anti-oxidative substances, this also suggesting no extreme sensitivity of the tulip bulb scale explants for oxidative stress.

Tulipaline content was determined in stalk explants and in regenerating and non-regenerating bulb scale explants. Surprisingly, tulipaline A and not its precursor tuliposide A was the most abundant compound in scale explants, although tulipaline was expected to be too reactive to occur in the free form in living cells. Therefore, it is likely that in bulb scales the tulipaline is present either in specialised vesicles or in an environment which reduces its reactivity. The amounts of tulipaline we found did differ strongly between stalks and bulb scales: the higher amounts of tulipaline in bulb scales might suggest a role in the lower regeneration potential but the comparison between regenerating and non-regenerating bulb scale explants did not confirm this suggestion. The tulipaline content changed during incubation; a clear decrease upon cutting the explants is seen, resembling a wounding effect. The content did increase later on during incubation of the explants indicating production of tulipaline A but

still no clear relation with vitality could be established. Furthermore, a comparison between initial tulipaline content and browning response for different tulip cultivars, indicated a negative instead of a positive correlation between the two parameters. All these observations suggest that the low viability of tulip bulb scale explants is not directly related to a toxic effect of tulipaline A.

Since both tested hypotheses do not seem to be related to the poor viability of tulip bulb scale explants outside the period in which regeneration is possible, the cause of this problem has to be found elsewhere. Probably the gradual progress of senescence leads to a stage where no redifferentiation is possible. Therefore, the development of a method to arrest bulb material in a physiological state where regeneration is possible might be necessary for the of micropropagation of tulip via bulb scale explants.

Samenvatting

De traditionele vermeerderingsmethoden voor de tulp zijn erg langzaam, waardoor het 25-30 jaar duurt voordat een nieuw ontwikkelde cultivar met bijvoorbeeld een verbeterde resistentie tegen ziektes of met een nieuwe kleur en/of bloemvorm op de markt gebracht kan worden. Daarom wordt er gezocht naar alternatieven die deze periode kunnen verkorten; de toepassing van weefselkweektechnieken lijkt hierbij veelbelovend. Wel bestaan er echter nog een aantal problemen, vooral bij het gebruik van bolrokken als startmateriaal: de explantaten zijn alleen vitaal en in staat om scheuten (waarvan de nieuwe bolletjes geproduceerd worden) te regenereren in een beperkte periode van het jaar. Dit project was gericht op de opheldering van de fysiologische factoren die verantwoordelijk zijn voor dit verlies van vitaliteit en regenererend vermogen.

Twee hypotheses zijn onderzocht. In de eerste hypothese stond de rol van oxidatieve stress centraal. Als weefsels niet in staat zijn om de zuurstofradicalen te verwerken die geproduceerd worden bij het snijden van het explantaat of niet in staat zijn om de oxidatieve zuurstof stress tijdens de incubatie te compenseren, kunnen ze zwaar beschadigd raken ten gevolge van processen die gerelateerd zijn aan oxidatieve stress zoals membraanschade, productie van toxische stoffen (MDA of fenolen) en beschadiging van DNA of eiwitten. Deze processen kunnen uiteindelijk leiden tot verlies van vitaliteit en de dood van de explantaten. In onze tweede hypothese hebben we de rol van tulipaline A (α-methyleen-γ-butyrolacton) in het vitaliteitsverlies onderzocht. In het verleden is het voorkomen van tulipaline A in verband gebracht met de resistentie van tulp tegen schimmelinfecties van bolrokken. Tulipaline A zou hierbij giftig zijn voor de schimmel bij verwonding van het tulpenweefsel (bij het binnendringen van de schimmel) of de voortgang van de infectie stoppen door het gelocaliseerd laten afsterven van de cellen rondom de infectieplaats. Omdat hierbij verbruining van het weefsel optreedt was de tweede hypothese dat de productie en/of het vrijkomen van tulipaline ook toxische effecten op tulpenweefsel zou kunnen hebben, wat uiteindelijk zou leiden tot vitaliteitsverlies en dood van de explantaten.

Om de hypothese van een extreme gevoeligheid voor oxidatieve stress te testen zijn verschillende strategieën gevolgd. In de eerste plaats werden fysiologische

parameters in regenererende tulpenexplantaten (bolrok en stengel) vergeleken met die in niet-regenererende bolrokexplantaten. In de tweede aanpak zijn verschillende weefselkweekcondities aangelegd (verschillende zuurstofcondities en toevoeging van anti-oxidatieve stoffen aan het weefselkweekmedium) waarbij fysiologische parameters gemeten zijn die betrokken zijn bij de zuurstofstress (bijv. veranderingen in enzym activiteit (lipoxygenase, SOD, peroxidases, catalase, PAL en polyfenoloxidase), membraansamenstelling en productie van MDA en fenolen). Om een indicatie te krijgen van de potentiële rol van veranderde zuurstofomstandigheden is verder een vergelijking gemaakt met andere weefselkweeksystemen (lelieschubben en appelstengelschijfjes). Dit stelde ons tevens in staat om de ontwikkelingsfase waarin de veranderde omstandigheden het meeste effect hebben te bepalen.

Er werden geen significante verschillen tussen de fysiologische reaktie van regenererende en niet regenerererende bolrokexplantaten gevonden. Tevens was het patroon van de reakties vergelijkbaar met het patroon dat gevonden werd in goed regenererende stengelexplantaten van de tulp. Enkele verschillen werden gevonden na een incubatie van 2-3 weken; deze verschillen zijn waarschijnlijk veroorzaakt door de lage vitaliteit van de bolrokexplantaten en niet de oorzaak van deze lage vitaliteit; de start van de regeneratie vindt plaats voordat de verschillen in fysiologische reactie worden gevonden. Uit de experimenten met gewijzigde zuurstofconcentraties waarbij 100 % of 2 % zuurstof werd gebruikt om respectievelijk de zuurstofstress te verhogen of te verlagen werd ook geen indicatie voor een extreme gevoeligheid voor zuurstofstress van bolrokweefsel gevonden. Regeneratie werd niet verbeterd door toepassing van laag zuurstof condities en de vitaliteit werd niet sterk negatief beinvloed door hoge zuurstof concentraties. Hoge zuurstof concentraties lijken een positief effect te hebben op de groei van de regenererende structuren nadat deze gevormd zijn maar hebben een klein negatief effect of de vorming van primordia. Geen duidelijke verbetering van de vitaliteit werd gevonden na de toevoeging van antioxidantia; ook dat duidt aan dat tulp bolrokexplantaten niet extreem gevoelig zijn voor oxidatieve stress.

Bij bepaling van het tulipaline gehalte van regenerende en niet-regenererende bolrokexplantaten bleek verrassenderwijs dat tulipaline A en niet de precursor tuliposide A het meest aanwezig was in de explantaten terwijl aangenomen was dat tulipaline A te reactief is om vrij voor te komen in levende cellen. Daarom is het

waarschijnlijk dat tulipaline in gespecialiseerde compartimenten voorkomt of in een omgeving waarin de reactiviteit beperkt is. De hoeveelheden tulipaline die gevonden werden verschilden sterk tussen bolrok en stengelweefsel. De hogere hoeveelheden tulipaline in bolrokken zouden een rol in de slechtere regeneratie kunnen spelen maar de vergelijking tussen regenererende en niet-regenererende bolrokexplantaten bevestigen dit niet. Het tulipaline gehalte veranderde tijdens de incubatie; een daling direct na het snijden van de explantaten werd gevonden: deze daling lijkt op een wond reactie. Dit wordt gevolgd door een geleidelijke toename tijdens de incubatie hetgeen wijst op een productie van tulipaline A maar geen duidelijke relatie met vitaliteit kon worden vastgesteld. Een vergelijking van het oorspronkelijke tulipalinegehalte en de verbruining van verschillende cultivars gaf een negatieve inplaats van de verwachte positieve correlatie tussen beide parameters te zien. Al deze waarnemingen suggereren dat de slechte vitaliteit niet direct is gerelateerd aan een toxisch effect van tulipaline A.

Omdat beide geteste hypotheses geen verklaring geven voor de slechte vitaliteit van tulp bolrokexplantaten buiten de periode waarin regeneratie mogelijk is, moet waarschijnlijk de oorsprong van deze problematiek elders gezocht worden. Mogelijk leidt het geleidelijk voortschrijden van de veroudering van het weefsel tot het bereiken van een situatie waarin geen regeneratie meer mogelijk is. Daarom lijkt de ontwikkeling van een methode waarbij de veroudering van bolrokmateriaal wordt geblokkeerd en het weefsel in een fysiologische stadium blijft waarbij regeneratie nog mogelijk is,. de meest wenselijke strategie om vermeerdering van tulp via weefselkweek vanuit de bolrok mogelijk te maken.

Curriculum vitae

Maarten van Rossum werd op 7 september 1968 geboren te Tiel. In 1985 werd de HAVO opleiding voltooid aan het Pax Christi College te Druten. In datzelfde jaar werd begonnen aan een HLO-opleiding (Biotechnologische afstudeerrichting) aan de toenmalige HMLS te Oss. Deze opleiding werd afgerond in 1989. Aansluitend werd de studie biologie begonnen aan de Katholieke Universiteit Nijmegen. In maart 1992 werd het doctoraal examen behaald met als hoofdvak experimentele plantkunde (welk vak werd gesplitst in een deel op de vakgroep en een deel op het CPRO-DLO) en als bijvak experimentele dierkunde. In dezelfde maand is begonnen aan het AIO-project op de vakgroep Plantenfysiologie van de Landbouwuniversiteit Wageningen waarvan de resultaten beschreven zijn in dit proefschrift.

List of Publications:

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