Physiology of mycelial growth of the mycorrhizal mushroom *Cantharellus cibarius* Fr.

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 31 januari 1986
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

1 De isolatie in reincultuur van cantharelmycelium uit vruchtlichaamweefsel wordt vooral bemoeilijkt door de aanwezigheid van een microflora in dit weefsel. (dit proefschrift)

2 De verwachting dat de cantharel, als mycorrhizavormende schimmel, zeer specifieke voedingseisen heeft om optimaal te kunnen groeien, blijkt niet gegrond. (dit proefschrift)

3 Er wordt bij het in reincultuur brengen van biotrofe schimmels te weinig rekening gehouden met mogelijke koolzuurbehoeften en met gasuitwisseling.

4 De grote gevoeligheid van cantharelmycelium in vitro voor allerlei toevoegingen aan het voedingsmedium, heeft in de natuur zijn parallel in de enorme achteruitgang van deze paddestoel.

5 Terecht wordt de mogelijkheid om de achteruitgang van de cantharel te bestrijden door het afvoeren van strooisel, of zelfs het afplaggen van de bodem, niet benadrukt door Jansen e.a.
Jansen, E. e.a. (1985). Wetensch. meded. KNNV 167: 59.

6 Het mechanisme van kiemrust van champignonsporen, beschreven door Rast e.a., zou opnieuw bestudeerd moeten worden, omdat als uitgangspunt de niet eenduidige resultaten van Lösel over een remmend effect van koolzuur op kieming zijn gebruikt.
Lösel, D.M. (1964). Annals of Botany 28: 541.
Rast, D. e.a. (1976). Mushroom Science 9 (1): 59.

7 De voeding van het rund en van de champignon hebben sterke analogïen.

8 In het licht van de uitspraak, dat de levensvormen op aarde te herkennen zijn aan de daarbij horende nematodenfauna, is het vreemd dat er geen systemen ontwikkeld zijn, gebaseerd op het al dan niet voorkomen van nematodensoorten, die de kwaliteit van het milieu karakteriseren.
Cobb, N.A. (1915). Yearbook USDA 1914: 457.

9 GLP (goede laboratoriumpraktijk) zou een verplicht onderwijselement voor de natuurwetenschappelijke richtingen aan de nederlandse universiteiten en hogescholen moeten zijn.
OESO, zie Nederlandse Staatscourant (1985) 144: 7.

10 De nederlandse champignons: champions.

Stellingen bij het proefschrift van G. Straatsma:
'Physiology of mycelial growth of the mycorrhizal mushroom Cantharellus cibarius Fr.'. Wageningen, 31 januari 1986.

INHOUD

ALGEMENE INLEIDING

Onderzoek aan eetbare paddestoelen

Het in dit proefschrift beschreven onderzoek over axenische groei van mycelium van Cantharellus cibarius Fr. houdt verband met onderzoek naar de teelt van eetbare paddestoelen.

De champignonteelt stamt uit het eind van de 17e eeuw, toen men in Frankrijk verse paardemest ging inoculeren met door champignonmycelium geïnfecteerde oude mest (Treschow, 1944). De teelt gebeurde in de open lucht.

Aan het eind van de vorige eeuw werd voor het eerst zogenaamd broed bereid met behulp van reïncultures, ontstaan uit gekiemde champignonsporen. Later werd ook geprobeerd een bijdrage te leveren aan de kennis over de voedingseisen van champignonmycelium, door mycelium onder axenische omstandigheden te bestuderen. Een voorbeeld hiervan is het onderzoek van Treschow (1944). Het axenisch onderzoek heeft tot nu toe niet veel bijgedragen aan de kennis over de groei van champignonmycelium in compost. Dit hangt samen met de afbraak van lignocellulose in compost (Gerrits e.a., 1967). Deze afbraak was tot voor kort moeilijk experimenteel te onderzoeken. Het in vitro onderzoek heeft tot nu vrijwel niet geleid tot fundamentele inzichten in de vruchtlichaamvorming van de champignon. Axenische technieken worden met succes toegepast in de veredeling (Fritsche, 1981). De ontwikkeling van de champignonteelt is tot nu toe vooral gebaseerd op empirie. Het fundamentele onderzoek heeft vooral verklaringen achteraf gegeven; een uitzondering is het fytopathologisch werk. Momenteel krijgt het biologisch onderzoek aan champignons in Nederland meer aandacht: moleculair-genetische technieken worden aangewend in de veredeling, er wordt meer onderzoek gedaan aan vruchtlichaamvorming en zeer onlangs is een fundamenteel onderzoek over het composteringsproces van start gegaan.

Programma voor domesticatie van de cantharel

De aanvang in 1980 van een onderzoeksproject met als titel "Fysiologie van de myceliumgroei van de cantharel" (Agrep, 1982) vloeide voort uit het beleid van de Directie Akkerbouw en Tuinbouw van het Ministerie van Landbouw en Visserij inzake de nederlandse champignonteelt. Dit beleid wordt onder andere ingegeven door de notie dat deze bedrijfstak kwetsbaar is door haar beperkte produktsortiment. Het is wenselijk om andere soorten eetbare paddestoelen, waarvoor een markt in Europa aanwezig is, te kunnen telen. Daartoe

behoren naast oesterzwammen, de japanse shii-take en inktzwammen zeker ook cantharellen en boleten (Nota, 1981). De europese markt voor cantharellen en de eveneens belangrijke boleten (eekhoorntjesbrood) en truffels is van aanzienlijke omvang. De structuur van deze markt is complex (Schaper, 1983).

Een eerdere poging in Nederland om een aanzet te geven tot domesticatie van de cantharel is gestrand op moeilijkheden rond het steriel kweken van mycelium (Schouten & Waandrager, 1979). Tijdens deze poging is duidelijk geworden met hoeveel scepsis claims met betrekking tot het kweken van mycelium in reincultuur (en domesticatie!) moeten worden bekeken.

Naar alle waarschijnlijkheid is de cantharel een mycorrhizavormende schimmel (Trappe, 1962; Froideveaux, 1975). Het bewijs hiervoor, door de zogenaamde postulaten van Koch te volgen, is nog niet geleverd. Tot nu toe zijn geen cultures van de cantharel uit mycorrhiza geïsoleerd en er zijn ook geen succesvolle infectieproeven gedaan. In ecologisch opzicht worden de mycorrhizavormende schimmels onderscheiden van saprofietische. De reden daarvoor is dat ze als wortelsymbionten rechtstreeks afhankelijk zijn van de stofwisseling van hun gastheer (Harley & Smith, 1983). Verscheidene saprofieten zijn onder kunstmatige omstandigheden te telen (Chang & Hayes, 1978); dit is tot nu toe met geen enkele mycorrhizavormer gelukt. De cultuur van de mycorrhizavormende truffels in Frankrijk en Italië (Delmas, 1978) en van de matsu-take in Japan (Tominaga, 1978) bestaat in feite uit beheersmaatregelen in terreinen waarin deze soorten van nature voorkomen. Plantages van gastheerboomsoorten in symbiose met cantharelmycelium naar analogie van zogenaamde truffelplantages bestaan niet. Het wordt als niet-relevant beschouwd om een dergelijk systeem te willen ontwikkelen. Deze ontwikkeling zou bovendien stuiten op vele ecofysiologische problemen rond kolonisatie en concurrentie in de rhizosfeer.

Omdat mycorrhizavormende schimmels als lastig te manipuleren schimmels bekend staan - van de cantharel was dat inmiddels wel bekend (Schouten & Waandrager, 1979) - is een grondige aanpak noodzakelijk om tot domesticatie te komen. De strategie in het domesticatieprogramma bestaat uit drie stappen. De eerste stap is het in reincultuur brengen van het mycelium en de vaststelling van de voedingseisen van mycelium in vitro. Na succesvolle afsluiting van dit project is de volgende stap de vruchtlichaamvorming. Tenslotte moet naar de praktijktoepassing gestreefd worden.

In dit proefschrift wordt de eerste stap in het domesticatieprogramma beschreven.

Isolatie en reincultuur van de cantharel

Bij de start van het onderzoek in 1980 was het Fries (1979) juist gelukt om reincultures te verkrijgen uit sporen van de cantharel. Wij kregen de beschikking over een van zijn stammen, namelijk nummer 740-b (ons nummer S1). In 1980 werd het sporekiemingswerk van Fries gereproduceerd en uitgebreid; in 1981 werden ook cultures rechtstreeks uit paddestoelweefsel gemaakt. Het vaststellen van de identiteit van een reincultuur waarin geen vruchtlichamen ontstaan is een principiëel probleem. Gezien de vroegere problemen rond de identiteit van "cantharel" reincultures (Schouten & Waandrager, 1979), werd gezocht naar een strikt criterium voor de identiteitsbepaling.

De collectie reincultures, waarvan de aanleg beschreven is in Hoofdstuk 1, dient als basis in het verdere domesticatieonderzoek. De biologische variabiliteit tussen de stammen kan vastgesteld worden en daarvan kan gebruik worden gemaakt.

Voedingsbehoeften van de cantharel

De reincultures van Fries groeiden erg langzaam (Fries, 1979); deze eigenschap hadden de door ons geïsoleerde stammen ook. Daarom stond de wens naar een snellere groei bij de studie naar de voedingseisen van cantharelmycelium centraal. Het medium en de incubatie-omstandigheden waarmee Fries (1979) cantharelmycelium isoleerde en met succes overentte, voorziet enigermate in de behoeften van cantharelmycelium. Dit medium (Fries, 1978, 1979) diende dan ook als basis in het verdere onderzoek. Het bestaat uit een oplossing van glucose, mineralen - met onder andere ammonium als stikstofbron - en vitaminen en voorziet daarom in de (primaire) behoeften van (heterotrofe) schimmels. De veronderstelling dat de cantharel een mycorrhiza-vormende schimmel is, doet vermoeden dat specifieke voedingseisen samenhangen met de symbiose met wortels. Het verbeteren van het voedingsmedium, in de zin van het toedienen van mogelijke groeifactoren, werd daarom in het bijzonder gericht op het effect van levende wortels in het voedingsmedium. Dit naar aanleiding van de onderzoeksresultaten van Melin (1963) over een bijzondere groeifactor die door wortels zou worden afgescheiden. Daarom werd enerzijds een medium, gebaseerd op de voedingsoplossing van Fries, geöptimaliseerd en anderzijds met behulp van dit medium een mogelijk wortel effect bestudeerd. In dit verband is het nuttig te onderstrepen dat wortels niet alleen fotosyntheseprodukten aan de mycorrhizaschimmel leveren (Melin & Nilsson, 1957; Harley & Smith, 1983), maar eventueel ook aminozuren, vitaminen en andere

algemene metabolieten (Melin, 1963). Daarnaast zou de wortel nog een specifieke groeistimulerende rol kunnen spelen.

In de loop van het onderzoek werd duidelijk dat een adequate wiskundige beschrijving van de groei noodzakelijk was. Hierdoor werd het mogelijk onderscheid te maken tussen lag-(aanpassings-)fase, relatieve groeisnelheid en maximum opbrengst (Griffin, 1981).

In Hoofdstuk 2 wordt verslag gedaan van de pogingen om het Friesmedium en de incubatie omstandigheden te optimaliseren. In Hoofdstuk 3 wordt het effect van levende wortels op de groei van de cantharel beschreven. In 1983 werd duidelijk dat de groeifactor(en), afgescheiden door wortels, vervangen kon(den) worden door koolzuur. Dit is beschreven in Hoofdstuk 4. In Hoofdstuk 5 tenslotte wordt ingegaan op de rol van koolzuur in het metabolisme van de cantharel.

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A STRAIN COLLECTION OF THE MYCORRHIZAL MUSHROOM CANTHARELLUS CIBARIUS OBTAINED BY GERMINATION OF SPORES AND CULTURE OF FRUIT BODY TISSUE.

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SUMMARY

Germination of spores of Cantharellus cibarius was obtained by five different methods finally resulting in the establishment of 12 pure strains. Living tomato roots were useful as a stimulant for germination although germination frequency remained low.

Although the preparation of pure cultures from fruit body tissue was technically not difficult they were usually heavily contaminated. Using modified Murashige & Skoog medium, 18 pure mycelial cultures from different origin were established; two were established on modified Fries medium.

Unambiguous evidence that the mycelial cultures were derived from C. cibarius was obtained with the aid of DNA hybridization experiments. These studies also revealed that C. cibarius is very slightly related to Craterellus cornucopioides, Stropharia rugosoannulata and Pleurotus cornucopiae, respectively. No base sequence homology was noted between C. cibarius and the cultivated mushroom Agaricus bisporus.

INTRODUCTION

Cantharellus cibarius Fr. is an ectomycorrhizal fungus which grows in intimate association with a large number of trees (Trappe, 1962). Detailed information on the specific nature of the association between fungus and host tree is lacking. Because of its economic value as an important edible mushroom, a number of investigators have tried to domesticate C. cibarius.

Although several successful attempts at isolating pure cultures from fruit body tissue have been claimed, there exist serious doubts whether they truly represent C. cibarius mycelia (Schouten & Waandrager, 1979). A simple method for the germination of spores of C. cibarius at very low frequency was described by Fries (1979).

In this paper the first phase of an attempt to domesticate C. cibarius is described.

Biological variability of different mycelial isolates will most likely greatly influence the response of mycelium to stimuli for fructification. Therefore establishment of a collection of mycelial cultures from C. cibarius samples of different geographical origin seems a prerequisite for further attempts at fruit body formation and ultimately for domestication.

Pure cultures can be obtained either by spore germination followed by cultivation of the germling(s), or by cultivation of explanted fruit body tissue. As germinating spores could already be a selected group of the total population, several methods to increase spore germination frequency were studied. Spore germination in mycorrhizal fungi is generally dependent on stimulation by living organisms (Fries, 1983). Underlying mechanisms are unknown, however. Spores of C. cibarius and of many other species can be stimulated by yeasts, especially Rhodotorula glutinis (Fries, 1943, 1979, 1983). The fungus Ceratocystis fagacearum was used to germinate spores of Lactarius species by Oort (1974), its activity was based on non-specified volatile substances. Tomato roots were used for Russula species by Melin (1962). Roots of host tree species were used for Thelephora terrestris, for Hebeloma species and for other species under less controlled conditions (Fries & Birreaux, 1980; Birreaux & Fries, 1981; Fries, 1981). Growing mycelium of the same species as the tested one were active for Leccinum species and Paxillus involutus, Fries (1981). All these stimulators were tested in the present study. Since agar media may contain growth inhibitory substances, addition of activated charcoal is necessary in many cases (Fries, 1978). For this reason liquid media were tested as well.

Since the growth characteristics of spore- and tissue-derived isolates of mycorrhizal fungi can be different, as demonstrated by Oort (1977) for Lactarius quietus, isolates were also made from fruit body tissue. Culture of fruit body tissue of C. cibarius is especially hampered by the presence of an associated microflora (Modess, 1941; Schouten & Waandrager, 1979). Antibiotics could help to prevent growth of these microorganisms. On the

other hand, rapid mycelial growth would compete strongly with contaminating microorganisms. Therefore, tomato roots, which produce the growth-stimulating M-factor (Melin, 1963), were tested.

Unambiguous identification of the isolates was obtained by molecular hybridization of DNA from mycelial cultures with DNA isolated from fungal fruit bodies of different origin.

MATERIALS AND METHODS

Collection and handling of fruit bodies

Firm and light-coloured specimens were collected in nature. They were transported in paper tissue and usually cultured on the day of collection. Some specimens were dried at 40 °C and deposited in the dried specimen collection of the Rijksherbarium at Leiden (L). Collections in the Netherlands were made in cooperation with Mrs E. Jansen of the Rijks Instituut voor Natuurbeheer at Arnhem and with Mrs M.H. Vaandrager, Mr J. Schreurs and Mr H. Wichers. A Canadian collection was obtained through Dr A.J.P. Oort. A list of collections which resulted in pure cultures is presented in Table 1.

Media and incubation

Cultivation of mycelium was performed in a Fries (F) medium (Fries, 1978), modified by replacing microminerals with those used by Von Arnold & Erikson (1977) in half dilution. Malt extract was omitted as in Fries (1979). The medium was either used as a filter-sterilized liquid or solidified with autoclaved 1.2% (w/v) agar.

Tomato root cultures were grown in a modified Murashige & Skoog (M&S) medium (Murashige & Skoog, 1962). Sucrose was used at a concentration of 20 g l^{-1} and vitamins were added as described by Meredith (1979). The medium was sterilized by autoclaving. Cultures were grown in the dark, mycelia at 20 °C and roots at 27 °C.

Tomato root culture

A clone of tomato roots (strain Panase F) was cultured by methods outlined by Butcher (1980). For experiments with spores and tissue of *C. cibarius*, root tips were cultured in M&S medium for 7 days before use.

Antibiotics

Because of the continuous presence of bacteria in fruit body tissue, penicillin and streptomycin (50 mg l^{-1}) were routinely used; benomyl (10 mg l^{-1}) was used to suppress ascomycetous contaminants.

Establishment of cultures

Isolation by means of spore germination

Fruit body caps were fixed to Petri dish lids with vaseline and placed over dish bottoms. Spores fell freely on to the agar medium, or into empty dishes, to be suspended in water for further handling. Cultivation in liquid medium was carried out in 100 ml flasks containing 25 ml of medium. Agar cultures were prepared in 9 cm Petri dishes containing 20 ml medium. After inoculation Petri dishes were sealed with "parafilm" to prevent desiccation. Usually, 10^5 to 2x 10^6 spores were addded.

Various methods were used to stimulate germination.

(a) Co-cultivation with the yeast Rhodotorula glutinis (Fres.) Harrison on agar medium with the addition of activated charcoal as described by Fries (1979).

(b) Co-cultivation with R. glutinis in liquid medium. R. glutinis was placed inside Visking dialysis tubing (25 mm width) in order to separate it from spores. As the tubing was sensitive to digestion by yeast extracellular enzymes, it was removed after two weeks. Activated charcoal was added.

(c) Co-cultivation with Ceratocystis fagacearum (Bretz) Hunt in liquid medium. C. fagacearum was placed in a small glass tube, with potato dextrose agar, inside the culture flask. Only volatiles can affect germination in this way. This is a modification of the method of Oort (1974). The experiment was carried out in the presence or absence of activated charcoal.

(d) With a living tomato root in free contact with liquid medium and spores (Melin, 1962). The experiment was carried out in the presence or absence of activated charcoal.

(e) Mycelium of an already established strain from N. Fries (number 740 b) was tested in liquid medium. It was separated from spores by a 0.2 µm cellulose nitrate membrane filter glued to a glass cylinder with silicone glue.

(f) As controls for the procedures described, cultivation was also carried out in flasks containing liquid medium with activated charcoal and pieces of dialysis tubing.

(g) A completely different method, resembling the mycorrhizal synthesis method of Marx & Ross (1970), was also used. Spores were inoculated on to roots of one-month-old axenic seedlings of Betula pubescens. The seedlings were grown on filter paper strips in glass tubes to which a small amount of the medium of Krupa & Fries (1971) was added. After three months the seedlings were transferred to F agar medium.

Spore germination on agar medium was inspected microscopically. In liquid medium, only yellowish fungal colonies, presumably of C. cibarius origin, were examined.

Isolation from fruit body tissue

Small tissue explants (about 3x3x3 mm) were cut with a scalpel from fracture planes made by hand cleavage of clean fruit bodies. Three or more explants were transferred to one 9 cm Petri dish. For growth stimulation, tomato roots were included in a number of dishes. Seven day-old root cultures were either placed on F medium, on which no further root growth occurs, or on M&S medium, which supports root growth. In addition, explants were either placed directly on to roots or some distance away to allow distinction between diffusible and non-diffusible growth factor(s) from the roots (Melin, 1963). Cultures were checked routinely for contaminating micro-organisms.

Maintenance of culture collection

Isolates were subcultured every four to six weeks in liquid medium. Borosilicate glass tubes of 27 mm diam were used, with 10 ml of medium. As frequent subculturing is time-consuming and can affect the genetic properties of strains (Fritsche, 1981), cultures were grown on agar slants of 16 ml in tightly sealed tubes and stored at 10°C. After one year, subcultures were made in liquid medium and, after successful growth, again transferred to agar slants. For each strain this was done in triplicate.

Identification of isolates

Tentative identification of C. cibarius was usually performed by making use of criteria such as similarities of colour and fragrance between isolates and fruit bodies and of cultural similarities between our isolates and Fries' polysporous C. cibarius strain 740b.

Definite identification was done using dot-blot DNA hybridization, in which the DNA was isolated from repeatedly washed mycelium and/or fruit bodies.

DNA isolation

DNA was isolated from fresh fruit bodies or washed mycelium by homogenization in a Waring blendor for 1 min at 4°C in the presence of equal volumes of STE-buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM EDTA) containing 0.5% SDS, and STE-saturated phenol. After centrifugation the water phase was collected and re-extracted twice with an equal volume of phenol-chloroform (1 : 1, v/v). Nucleic acid was then precipitated by the addition of two volumes of ethanol in the presence of 200 mM LiCl. After drying, the precipitate was dissolved in 10 mM Tris-HCl, pH 7.2 and 150 mM NaCl and incubated in the presence of 10 $\mu g\ ml^{-1}$ RNase A (Sigma, type III-A) for 60 min at 37°C.

DNA was then further purified by two successive extractions with equal volumes of STE-saturated phenol followed by an extraction with an equal volume of phenol-chloroform and extraction with an equal volume of chloroform-isoamylalcohol (24 : 1, v/v). Subsequently the DNA was precipitated by the addition of 2.5 volumes of ethanol in the presence of 300 mM Na-acetate and 10 mM Mg-acetate (pH 6.0). DNA was recovered by centrifugation and after drying under vacuum dissolved in STE-buffer at a final concentration of 0.5 $mg\ ml^{-1}$.

Application of DNA to cellulose filters

Prior to application to the filters (Schleicher and Schüll, BA 85), DNA was denatured by addition of one-fifth volume of 2N NaOH followed by heating for 5 min at 80°C. After denaturation the DNA solution was rapidly chilled on ice and diluted with an equal volume of 2M NH_4-acetate. Subsequently the DNA solution was diluted with 1M ice-cold NH_4-acetate to final DNA concentrations of 0.25, 0.025 and 0.0025 $ug\ \mu l^{-1}$. A 1 μl sample of each dilution was spotted on pre-wetted nitrocellulose filters. After drying in air the filters were baked at 80°C for 2 h in a vacuum oven.

Preparation of ^{32}P-labelled DNA Probes

The ^{32}P-labelled DNA probes were prepared by nick-translation (Maniatis et al., 1982). In short: to 1 μg of DNA, 5 μl of 10 x nick-translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1mM dithiothreitol, 500 $\mu g\ ml^{-1}$ bovine serum albumin (Pentax fraction V)), 1 μl of a solution of the deoxynucleotide triphosphates dGTP, dTTP, and dCTP (2mM each), 2 μl of ^{32}P-labelled dATP ($3000\ Ci\ mmol^{-1}$; $0.01\ mCi\ \mu l^{-1}$; Radiochemical Centre, Amersham), 0.5 μl DNase

I (0.1 μg mg^{-1}; Worthington Biochemicals), and 5 units of E. coli DNA polymerase I (Boehringer Mannheim) were added. After thoroughly mixing, the reaction mixture was incubated for 60 min at 16°C. The reaction was stopped by the addition of 2 μl of 0.5M EDTA followed by heating for 5 min at 70°C. The ^{32}P-labelled DNA was separated from unincorporated deoxynucleotide triphosphates by chromatography through a small column (1 ml) of Sephadex G50 (Pharmacia).

Dot-blot-hybridization

Prior to hybridization of the ^{32}P-labelled DNA probe to the different DNA preparations spotted on the nitrocellulose filters, the filter was pre-incubated for 2 hr at 42°C in a sealed plastic bag containing 150 μl cm^{-2} of pre-hybridization solution (50% formamide, 5 x Denhardt's solution (Denhardt's solution : 0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g bovine serum albumin in 500 ml water), 5 x SSPE-buffer (SSPE : 0.9M NaCl, 50 mM NaH_2PO_4, pH 7.4, 5 mM EDTA), 0.1% SDS and 120 μg ml^{-1} denatured salmon sperm DNA). After incubation the filters were blotted between several sheets of Whatman 3 MM filter paper and subsequently incubated (overnight at 42°C) in another plastic bag in the presence of hybridization buffer (50 μl cm^{-2}) to which the ^{32}P-labelled DNA probe was added (hybridization buffer : 50% formamide, 1 x Denhardt's solution, 5 x SSPE buffer, 0.1% SDS and 120 μg ml^{-1} denatured salmon sperm DNA).

After hybridization the filters were washed at room temperature three times with 50 ml of 6 x SSC (SSC : 0.15M NaCl, 15 mM Na-citrate, and 122 mM EDTA), followed by one wash with 100 ml of 5 x SSC. After washing the filters were blotted dry with Whatman 3 MM filter paper, and exposed to Kodak XAR films at -70°C with Du Pont Lightning-Plus intensifying screens.

RESULTS

In Table 1 a summary is presented of mycelial cultures established from spores and/or fruit body tissue prepared from fruit bodies gathered at different locations.

Table 1: Origin of *C. cibarius* pure cultures.

ORIGIN		DOMINANT TREE(S)	STRAIN NUMBERS, isolated from spores (s) or tissue (t)
Sweden		?	s: 1 1)
Canada, Quebec		?	s: 2
the Netherlands			
Province	Area		
Friesland	Elsloo	*Quercus robur*	s: 8 t: 6, 7
Drenthe	Diever	*Pinus sylvestris*, *Betula pubescens*	s: 3 t: 1
Drenthe	Zwiggelte 2)	*Q. robur*	t: 4, 5, 14
Drenthe	Hooghalen	*Q. robur*	t: 20
Drenthe	Spier	*Picea abies*	t: 3
Drenthe	Spier	*Q. robur*	t: 13
Drenthe	Hijken	*Q. Robur*	t: 2
Gelderland	Ugchelen	*P.sylvestris*, *Q.robur*	s: 4, 5, 6
Gelderland	Ugchelen	*Q. rubra*	s: 9
Gelderland	Bruggelen	*P.sylvestris*, *Q.robur*	s: 7 t: 15
Utrecht	Bunnik 3)	*Fagus sylvatica*	s: 10, 11, 12, 13 t: 8, 9, 10, 11, 12
Limburg	Griendtsveen	*Q. rubra*	t: 16, 17, 18, 19

1) strain 740 b of N. Fries
2) successful isolation in two successive years
3) *C. cibarius* var. alba

Cultivation by spore germination

Spore prints of approximately 40 fruit bodies were used for germination. However, many failed due to the presence of contaminating micro-organisms. In the Rhodotorula series it was found that the yeast easily swarmed off on the agar-surface, or digested dialysis membranes; this also led to several failures. Germination was found with four stimulation methods and also in the control. Only the method with already established mycelium as a stimulant failed to give positive results. After about three months primary cultures could be transferred. In liquid medium, cultures could be established both in the presence and the absence of activated charcoal. The use of tomato roots resulted in the highest number of successful primary cultures. The method of Marx & Ross (1970) resulted during the first incubation only in swollen spores, mycorrhizas were not formed; during the second incubation on F medium several non-mycorrhizal mycelia developed. Isolates made by each method were stored for further use.

In agreement with observations of Fries (1979), germination frequency was very low. Differences between replicates were high, many without any germination.

No attention was paid to the mono-, di- or polysporous origin of the cultures. Two strains in the collection were found to be clampless and could be monosporous.

No cultures could be derived from spores kept at 4°C during a period of 1 month.

Isolation from fruit body tissue

From 70 fruit bodies 20 pure cultures of C. cibarius were obtained. These 20 specimens belonged to 12 collections sensu Oort (1981); they originated from 10 different geographical sites in the Netherlands. The 20 originated from different media; a compilation is presented in Table 2.

In most cases isolated fruit body tissue turned out to be contaminated by bacteria and/or fungi. Contaminating organisms, presumably belonging to the natural microflora of C. cibarius fruit bodies, were identified as in Table 3. Non contaminated explants could be cultured. Primary cultures could be transferred after up to three months of incubation.

None of the antibiotics (penicillin, streptomycin, amikacin, azlocillin, ceftazidin or tobramycin) could stop growth of bacteria from explants on F medium if added separately. Nearly all cultures were established on M&S

Table 2: Number of cultures isolated from fruitbody tissue obtained on different media, in presence or absence of living tomato roots.

medium	root absent	root present	
		explant placed on root	explant placed apart from root
F + O	1		
F + P,S,B			1
M&S + P,S	3	1	6
M&S + P,S,B	2	1	3
Variable 1)	2		

Abbreviations
O = Oxytetracyclin
P = Penicillin
S = Streptomycin
B = Benomyl

F = modified Fries medium
M&S = modified Murashige & Skoog medium

1) explants incubated on different media during isolation effort.

Table 3: Organisms isolated from C. cibarius fruitbodies.

bacteria	Pseudomonas spp. (5 isolates)	1)
	Synechococcus spec.	2)
fungi 3)	Calcarisporium arbuscula Preuss	
	Chalara cf microchona W.Gams	
	Chloridium virescens (Pers. ex Fr.) W.Gams + Hol.-Jech.	
	Exophiala jeanselmei (Langer.) McGinnis & Padhye	
	Phoma cava Schulzer	
	Phoma viburnicola Oudem.	
	Septonema chaetospira var. pini Bourchier	
	Tetracladium spec.	
	cf Ustilago spec.	
	Verticillium insectorum (Petch) W.Gams	
	Mycelia sterilia (10 isolates, 2 clampless Basidiomycetes)	

Identification was done by
1. Dr H.J. Miller, Planteziektenkundige Dienst, Wageningen;
2. Dr G.M. Lokhorst, Rijksherbarium, Leiden;
3. Drs H.A. van de Aa, W. Gams, R.A. Samson, J.A. Stalpers, Centraal Bureau voor Schimmelcultures, Baarn.

medium, containing penicillin and streptomycin.

Living tomato roots did not have an effect on the establishment of cultures.

Characterization of C. cibarius isolates

The strains considered to be C. cibarius had many characteristics in common with the Fries strain. The growing mycelium was cream-coloured and turned yellowish-orange with ageing. After drying, mycelia were egg-yellow to orange. All these colours were also present in fruit bodies. The fragrance of cultures was very fruity, as were fruit bodies. Growth rates were low; doubling time of exponentially growing hyphal suspensions was two days or more (details to be published). Hyphae were clamped; exceptions were two strains obtained through spore germination.

Differences between strains were most pronounced with regard to their growth pattern on agar medium. Some strains grew with a dense, fine-stranded, aerial mycelium. Other strains stopped growth rather soon, resulting in a smooth lump of mycelium, with a darker, orange colour. Under laboratory conditions growth characteristics of several isolates seemed to change after repeated transfer. There are no differences between spore and tissue isolates as groups.

Dot-blot hybridization

To prove unambiguously that the mycelial cultures were indeed derived from spores or tissue of C. cibarius and not due to contamination of the cultivation media by spores and/or mycelium of other fungi, DNA from the cultures was isolated and labelled by nick-translation with radioactive phosphate. Subsequently the labelled DNA probe was hybridized to DNA prepared either from fruit bodies of C. cibarius collected in nature or from mycelia and/or fruit bodies of Craterellus cornucopioides (L.: Fr.) Pers., Agaricus bisporus (Lange) Imbach, Stropharia rugosoannulata Farlow ex Murrill and Pleurotus cornucopiae (Paul.) Rolland cultivated in our Station.

From the results obtained (Fig. 1) it could be concluded that the DNA prepared from the cultures hybridized to the DNA prepared from the fruit bodies of C. cibarius to the same extent as it did to its own. On the contrary, only weak hybridization was noted to the DNA prepared from mycelia and/or fruit bodies of C. cornucopioides, S. rugosoannulata and P. cornucopiae, while no detectable hybridization was found to the DNA prepared

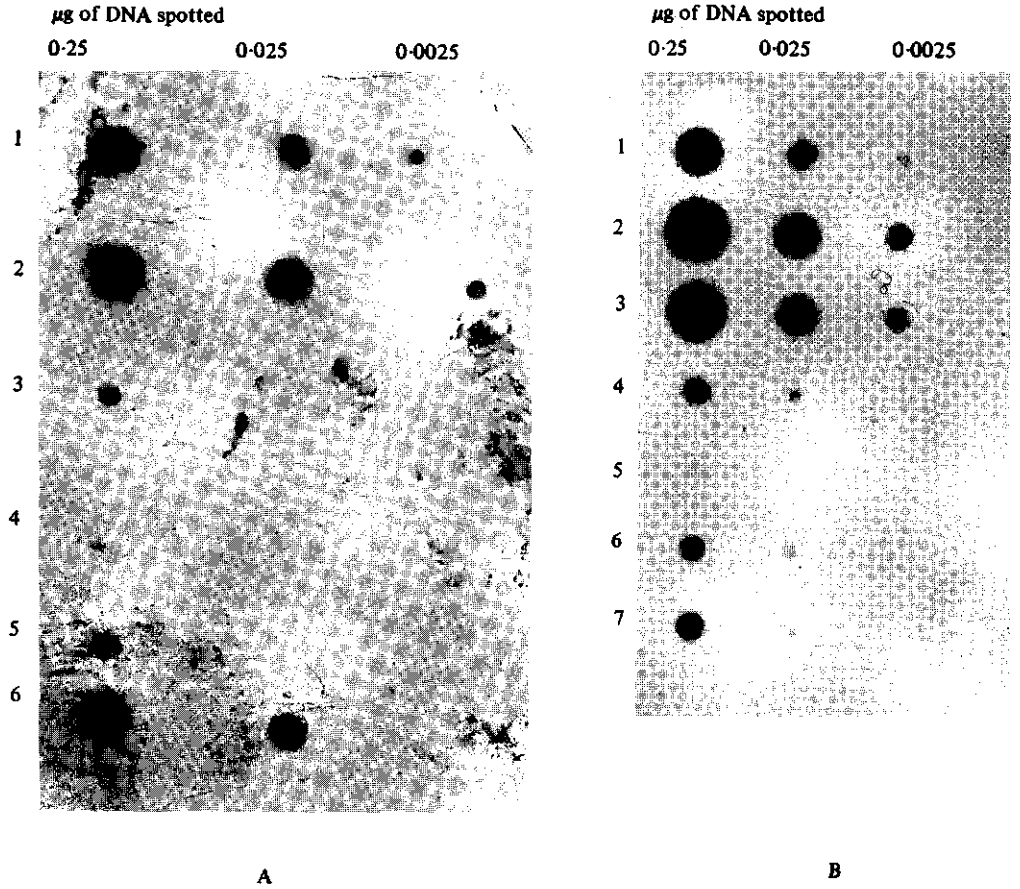

Figure 1: Autoradiograph of dot-blot hybridizations of nick-translated Cantharellus cibarius mycelium DNA to different amounts (0.25, 0.025, and 0.0025 µg) of DNA of various sources.
A. Nick- translated DNA of strain s1 (= Fries strain 740b) mycelium hybridized to DNA spots of the following: 1. C. cibarius mycelium (strain s1); 2. C. cibarius fruitbodies (Drenthe, Hooghalen); 3. C. cornucopioides mycelium; 4. Agaricus bisporus mycelium; 5. Stropharia rugosoannulata fruitbodies; 6.C. cibarius mycelium (strain t6).
B. Nick-translated DNA of strain t3 mycelium hybridized to DNA spots of the following: 1. C. cibarius mycelium (strain t3); 2. C. cibarius fruitbodies (Drenthe, Hooghalen); 3. C. cibarius fruitbodies (Limburg, Griendtsveen); 4. C. cornucopioides mycelium; 5. A. bisporus mycelium; 6. S. rugosoannulata fruitbodies; 7. Pleurotus cornucopiae fruitbodies.

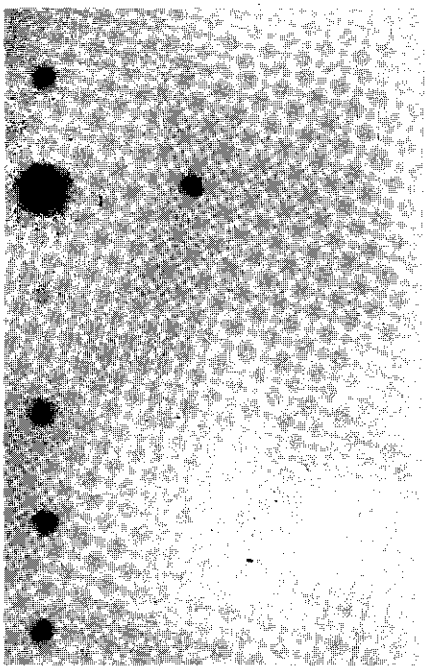

Figure 2: Autoradiograph of dot-blot hybridizations of nick-translated C. cornucopioides mycelium DNA to different amounts (0.25, 0.025, and 0.0025 µg) of DNA prepared from: 1. C. cibarius fruitbodies (Limburg, Griendtsveen); 2. C. cornucopioides mycelium; 3. A. bisporus mycelium; 4. S. rugosoannulata fruitbodies; 5. P. cornucopiae fruitbodies; 6. C. cibarius mycelium (strain s1).

from fruit bodies of A. bisporus. A negative control experiment has confirmed these observations in that a labelled DNA probe of C. cornucopioides hybridized strongly only to its own DNA while a weak or no hybridization was found to the DNA prepared from other mushroom species (Fig. 2). The same experiment was carried out with labelled A. bisporus mycelial DNA, the results were essentialy analogous.

Maintenance of culture collection

Our culture collection now contains 33 strains, 13 of which were obtained through spore germination (including the Fries strain). Maintenance on liquid medium presents no technical problem. Maintenance on agar at 10°C was good. In the second year two strains failed to grow; they were replaced from the liquid medium collection. Preliminary results suggest that liquid nitrogen storage may be successfully applied.

DISCUSSION

Pure cultures of C. cibarius were obtained by means of spore germination and by isolation from fruit body tissue. Spore germination frequency was very low. The work of Fries and co-workers shows that spores of many mycorrhizal fungi germinate at frequencies below 1% and with incubation times of more than one week in the presence of a non-specific germination stimulator such as yeast. Some species (Leccinum spp., Hebeloma spp., Thelephora terrestris, Paxillus involutus, Boletus luridus) show improved germination when a specific germination stimulator is used. These stimulators are host tree-roots or mycelium of the same species as the tested spores. The results strongly suggest that this stimulation of germination is a regulatory instead of a nutritional process, though no specific stimulator of spore germination has been identified. In view of the germination stimulation by such different organisms - R. glutinis, C. fagacearum, tomato roots - all resulting in only a very low germination rate, and by the long incubation period, germination of C. cibarius appears to be spontaneous and is stimulated unspecifically. In this study irregular germination was found between replicates. This effect may suggest an autocatalytic germination process (induction by mycelium) but our direct experiment with mycelium did not stimulate germination. On the basis of our experiments, tomato roots are the preferred germination stimulant.

Isolation from fruit body tissue was more successful in M&S medium than in F medium. Contamination problems on F medium were unsolvable. Since M&S medium is less suitable for growth of established pure cultures than F medium (to be published) the successful use of this medium may be due to suppression of growth of contaminating bacteria. The lower pH of the M&S medium was not the effective factor, since tissue explants on F medium of pH 4.2 all became heavily contaminated. The carbohydrate source in M&S medium (sucrose) could be effective since many Pseudomonads, which were present among the contaminating bacteria, are unable to metabolize this sugar. Presence of antibiotics in F medium had no effect.

To increase isolation success from fruit body tissue, further studies into growth prevention of the associated microflora of the tissue are required. The role of organisms associated with fruit body tissue is unknown. Some of the identified species are known as weak parasites and saprophytes, but fruit body induction cannot be excluded.

Although circumstantial evidence exists that the cultures are of C. cibarius origin, it is clear that in view of the presence of many different fungi in fruit body tissue, very strict identification standards should be met. The DNA hybridization figures show quite clearly that the different C. cibarius cultures used are genetically identical to fruit bodies of C. cibarius found in nature. It is interesting to note that Craterellus cornucopioides, Stropharia rugosoannulata and Pleurotus cornucopiae have a slight relationship with C. cibarius, whereas the cultivated mushroom, Agaricus bisporus, is not related at all (as judged from the lack of cross-hybridization). Similar observations have been made recently by other investigators (Horgen et al., 1984). The significance of these observations is unknown and will require detailed molecular analysis. However, such results emphasize the usefulness of this technique in discriminating between taxa.

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GROWTH REQUIREMENTS OF MYCELIAL CULTURES OF THE MYCORRHIZAL MUSHROOM CANTHARELLUS CIBARIUS FR.

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SUMMARY

The requirements of 2 strains of Cantharellus cibarius for mycelial growth in liquid medium were similar. A modified Fries (F) medium with 5 mM succinate or 25 mM MES as a buffer, an initial medium pH of 5.5, and incubation around 22 °C were optimal. The medium should be sterilized by filtration. Emersed growth was found to be better than standard, submersed incubation. Submersed growth was limited by production of staling substances. C. cibarius mycelium grows slowly and is very sensitive to various additions, but the basic growth requirements are similar to those of other mycorrhizal fungi.

The specific growth rates of both strains increased in the course of the study.

INTRODUCTION

The assessment of the growth requirements of mycelium of Cantharellus cibarius Fr. in vitro is a second step in the domestication of this mushroom, after successful isolation on modified Fries (F) medium and modified Murashige & Skoog (M&S) medium (Straatsma et al., 1985). Growth on F medium was better than on M&S medium, but still very slow, in accordance with an earlier report (Fries, 1979).

In this paper experiments are described on physical and chemical aspects of mycelial growth of C. cibarius using liquid F medium (pH 5.5) and an

incubation temperature of 20°C. This temperature was convenient for isolation purposes and preferable for growth of C. cibarius (Fries, personal communication). The chemically defined Fries medium is basically the modified medium of Nielsen (1930). It was modified and used by Fries (1938, 1948, 1976, 1978), Lindeberg (1941) and Melin (1946) for nutritional studies on different fungi. This Fries medium satisfies basic nutritional requirements of mycorrhizal fungi because it contains glucose, NH_4-nitrogen, several minerals, and vitamins (Molina & Palmer, 1982; Harley & Smith, 1983).

Considered general aspects are medium sterilization, incubation temperature, a comparison of different media commonly used in mycorrhizal studies, medium acidity, and water potential. Nutrition is studied by variations in minerals, carbon and nitrogen sources, and vitamins. Furthermore emersed incubation and limitations of submersed growth are described.

Duration of lag phase, specific growth rate, and maximum yield were taken as growth characteristics (Griffin, 1981).

MATERIALS AND METHODS

Strains

Experiments were done with strain S1 isolated by spore germination by N. Fries (his strain number 740b) (Fries, 1979), and with strain T6 isolated from fruitbody tissue (Straatsma et al., 1985). The strains were taken once a year from a collection held at 10°C. In a years period they were subcultured monthly under standard conditions and discarded at the end.

Media

Media were derived from the literature and from further modifications of the F medium (Table 1). pH was adjusted to 5.5 with 1 M KOH. Media were sterilized by filtration through 0.2 µm cellulose nitrate filters (Sartorius, Göttingen, F.R.G.). Sterility was checked after 2 days of incubation at 26°C. Glassware and other materials were sterilized by autoclaving for 15 minutes at 121°C.

Cultivation

Standard growth experiments were done in round-bottomed glass tubes with

an inner area of 4.9 cm^2, using 5 ml medium. As inoculum small mycelial tufts were cut from 3 to 5 weeks old colonies, dry weights varying between 0.02 and 0.1 mg. Tubes were closed with rayon fibre plugs (Utermöhlen, Amsterdam, the Netherlands) and incubated upright at 20°C in the dark. Cultures normally developed submersed. Emersed cultures were obtained by inoculating mycelial tufts either on pre-weighed, Whatman No. 1 filter paper bridges in liquid medium, or on medium solidified with autoclaved agar to a final concentration of 1.2% (w/v). Agar cultures were done either in tubes (standard), or in petri-dishes with 20 ml medium and 4 inoculum tufts. To facilitate yield determination, in some experiments the agar surface was covered with cellophane and tufts were inoculated on top of this. To prevent desiccation, petri-dishes were sealed with parafilm.

Experiments were done in 5 fold.

Yield determination

Usually dry weight was determined after about 20 days of incubation. Colonies were removed with tweezers, washed twice in water, dried on washed, pre-weighed filter paper disks (Whatman No. 1, 25 mm diam.) at 104°C for 1 hour, and incubated overnight over silicagel. Weights were determined with an accuracy of 0.1 mg. Average yield (Y) and standard errors were calculated.

Table 1. Composition of modified Fries (F) medium.

glucose	22.2	mM	KH_2PO_4	1.5	mM
$(NH_4)_2$-tartrate	5.4	mM	$MgSO_4$	0.41	mM
inositol	56	µM	NaCl	0.34	mM
nicotinamide	0.8	µM	$CaCl_2$	0.18	mM
4-aminobenzoic acid	0.7	µM	EDTA	25	µM
pyridoxine	0.5	µM	$FeSO_4$	25	µM
pantothenic acid	0.4	µM	$MnSO_4$	5	µM
thiamine	0.3	µM	H_3BO_3	5	µM
riboflavine	0.3	µM	$ZnSO_4$	5	µM
biotine	0.1	µM	KI	2.5	µM
			$NaMoO_4$	50	nM
			$CuSO_4$	5	nM
			$CoCl_2$	5	nM

RESULTS

Inoculated tufts often showed a prolonged lag phase or erratic growth. In many experiments average yields had high standard errors; therefore experiments were repeated until standard errors were below 25%.

The growth requirements of strains S1 and T6 were very similar. T6 had a greater tendency to react to inoculation with erratic growth, but once growth started it grew strongly. Differences in strain characteristics were not influenced by environmental manipulation.

During growth the pH of the medium decreased to a final value of 4.5. Growth on F agar, measured as hyphal expansion, was about 0.4 mm.day^{-1} (data after 5 years of subculture).

Growth curves

Growth under standard conditions is shown in Figure 1. Each strain was grown in 1982 and in 1984. Logistic functions (equations 1a, 1b) were transformed (equation 2) (Causton, 1978) and fitted by lineair regression.

$$Y_T = Y_{max} / (1 + C \times e^{-SGR \times T}) \quad \text{equation 1a}$$

$$C = (Y_{max} / Y_i) - 1 \quad \text{equation 1b}$$

$$\ln(Y_{max} / Y_T - 1) = - SGR \times T + \ln(C) \quad \text{equation 2}$$

Y_T: yield on time T, Y_{max}: maximum yield, C: constant, SGR: specific growth rate, T: time, Y_i: initial yield (effective inoculum).

Logistic functions are determined by 3 variables and therefore it was necessary to do cyclic lineair regression by step-wise varying maximum yield, until a maximum for the coefficient of correlation was found. For calculus, in correspondence with the visually observed lag phase of 4 to 6 days, inoculum dry weight was not used as Y_0 but as Y_4. Exponential growth occurs until $e^{-0.5}$ = 0.6 mg (Fig. 1). Under identical conditions both strains showed a higher SGR in 1984 than in 1982. The exponential growth phases ended after

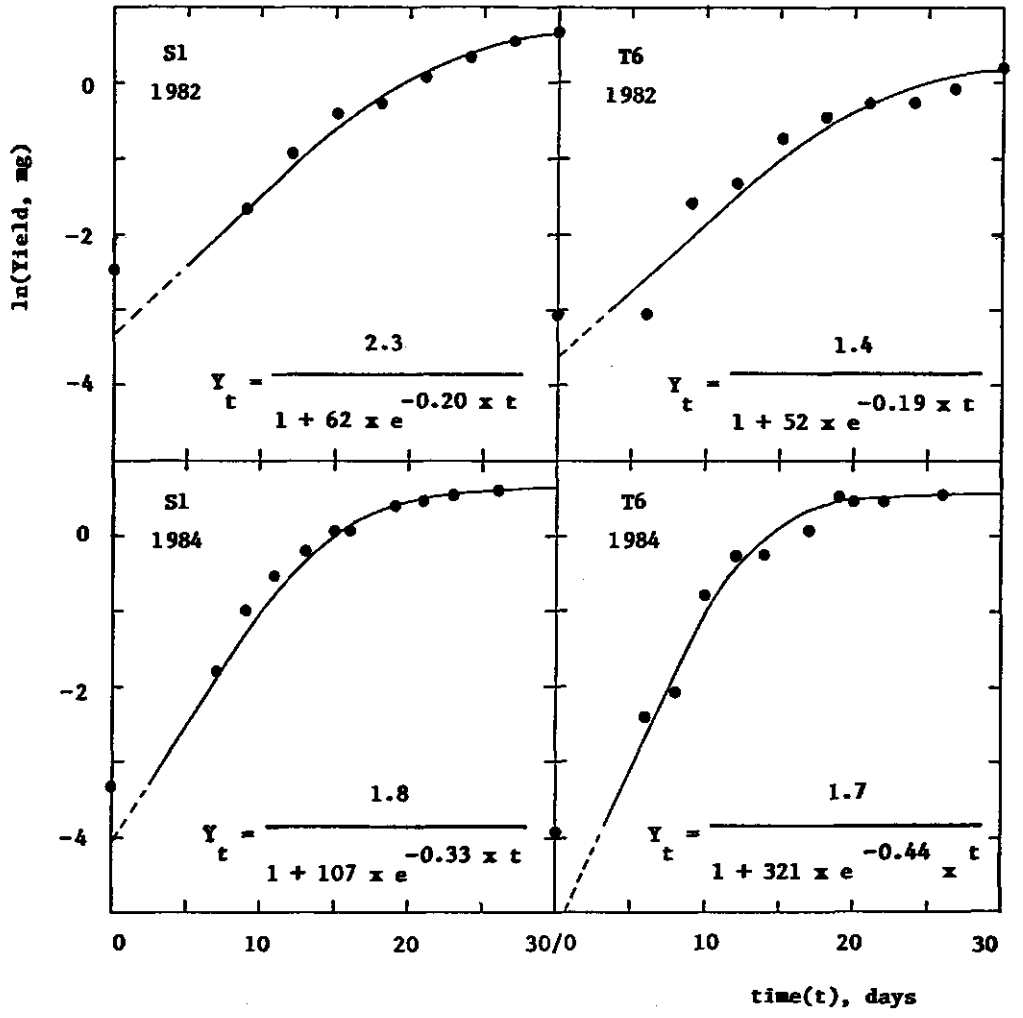

Figure 1. Fitted logistic functions of dry weight yields (Y) of strains S1 and T6 cultured in 5 ml liquid modified Fries medium (data collected in 1982 and 1984)

16 days in 1982 and after 12 days in 1984. Since the maximum yields in the presented curves are lower than some yields in other experiments, a general variance in maximum yield occurred but Y_{max} was limited to about 2 mg.

General conditions

Growth on a medium that was autoclaved after filter-sterilization was considerably inhibited as compared to growth on non-autoclaved medium.

Four temperatures from 16 to 26 °C were tested. Growth in F medium at 20 and 24 °C was better than at 16 or 26 °C (Table 2).

Table 2. Effects of different temperatures on yield after 21 days of incubation.

incubation temperature, °C	Yield, strain S1 Y, mg ± S.E.	Yield, strain T6 Y, mg ± S.E.
16	0.9 ± 0.3	0.8 ± 0.3
20 (standard)	1.7 ± 0.3	1.7 ± 0.2
24	1.6 ± 0.3	1.6 ± 0.1
26	1.0 ± 0.2	1.2 ± 0.1

Growth was tested in different semi-synthetic media (Molina & Palmer, 1982; Oort, 1981): Modess (Modess, 1941), MMN (Marx, 1969), and BAF (Moser, 1960). In these media, however, as well as in the defined, modified M&S medium (Straatsma et al., 1985), growth fell considerably behind that in F medium (Table 3). Also the addition of the usual concentrations of chemically ill-defined substrates such as yeast extract (100 $mg.l^{-1}$), malt extract (1 $g.l^{-1}$), casein hydrolysate (1 $g.l^{-1}$), pepton (1 $g.l^{-1}$), and corn steep liquor (1 $ml.l^{-1}$) failed to improve growth in F medium. With the exception of casein

Table 3. Yields on different synthetic and semisynthetic media after 21 days (strain S1), and 19 days (strain T6) of incubation at 20°C.

medium	strain S1 Y, mg ± S.E.	strain T6 Y, mg ± S.E.
F (standard)	1.4 ± 0.1	1.7 ± 0.2
MMN	0.7 ± 0.1	0.8 ± 0.2
BAF	0.9 ± 0.1	0.4 ± 0.1
Modess	0.3 ± 0.1	0.6 ± 0.1
M&S	0.5 ± 0.3	0.4 ± 0.2

hydrolysate they rather brought growth to an earlier termination.

Potassium salts of different buffers were tested and compared with the standard, ammonium tartrate buffer. Morpholino ethane sulfonic acid (MES), successfully applied by Giltrap & Lewis (1981), was used at 25 mM, phosphate at 10 mM, and succinate at 5 mM. Ammonium was added as 10.8 mM NH_4Cl. Succinate and MES proved excellent buffers giving a good growth and a modest pH shift (Table 4).

Table 4. Yields on media with different pH buffers and corresponding pH shift, after 20 days of incubation at 20°C.

pH buffer	strain S1		strain T6	
	Y, mg ± S.E.	Δ pH	Y, mg ± S.E.	Δ pH
5mM tartrate	1.5 ± 0.3	-0.8	1.7 ± 0.3	-0.8
5mM succinate	1.7 ± 0.1	-0.2	2.3 ± 0.4	-0.3
10mM phosphate	1.2 ± 0.2	-1.1	1.7 ± 0.2	-1.3
25mM MES	1.7 ± 0.1	-0.2	2.1 ± 0.7	-0.2
none	0.9 ± 0.1	-1.3	1.1 ± 0.1	-1.3
standard medium	1.3 ± 0.1	-0.7	2.2 ± 0.2	-0.9

Using 5 mM succinate as a buffer, a pH range of 3.5 to 6.5 was tested in steps of 0.5 units. Growth at the initial pH values of 5.5 and 6.0 was better than that at lower or higher pH values (Table 5). Final pH values shifted to pH 4.5.

Table 5. Yields, with corresponding pH shift, on media with different initial pH values, after 22 days of incubation at 20°C.

initial pH	strain S1		strain T6	
	Y, mg ± S.E.	Δ pH	Y, mg ± S.E.	Δ pH
3.5	0.2 ± 0.1	+0.3	0.3 ± 0.1	+0.3
4.0	0.9 ± 0.2	+0.1	0.9 ± 0.3	+0.1
4.5	0.7 ± 0.4	0	1.0 ± 0.2	0
5.0	1.4 ± 0.3	-0.1	0.9 ± 0.5	-0.1
5.5	1.5 ± 0.3	-0.2	1.8 ± 0.3	-0.2
6.0	1.7 ± 0.3	-0.4	1.8 ± 0.2	-0.3
6.5	1.3 ± 0.2	-0.6	1.2 ± 0.4	-0.4
standard medium	1.3 ± 0.8	-0.7	1.8 ± 0.3	-0.8

Experiments with additions of 0.4 M mannitol, and also of glucose and sucrose, resulting in a water potential shift from 0.11 to 1.08 MPa, always showed average yields with high standard errors. They left an impression of better (i.e. denser) growth, compared to growth on standard medium, of those tufts that overcame their lag phase normally. Considerable effects, however, were not found.

Nutrients

Mineral sets of F and M&S medium were compared. Both media support growth and are chemically defined. Macro minerals (N, P, K, Mg, Ca, and Na) and micro minerals (Fe, Mn, Zn, B, etc.) were tested separately. The media were buffered with 25 mM MES, 10.8 mM NH_4Cl replaced $(NH_4)_2$-tartrate in F macro nutrients. No significant differences were found between macro and micro mineral sets from F or M&S media. Growth on medium without micro minerals was good. Additions of sodium and potassium salts higher than 15 mM, resulting in a waterpotential of 0.14 MPa, were inhibitory to growth. Crystals were formed in F medium at and above 2 mM $CaCl_2$.

Carbohydrates were tested in concentrations equivalent to the standard 22.2 mM glucose. The standard nitrogen source, $(NH_4)_2$-tartrate, was present as a second carbon source. Possible inhibitory effects were studied by addition of the standard concentration of glucose to the tested carbon

Table 6. Growth on different carbon sources, in the presence of $(NH_4)_2$-tartrate, during 17 days (strain S1) and 19 days (strain T6) of incubation (* S.E. > 25%).

carbon source	strain S1		strain T6	
	no addition Y, mg	with glucose Y, mg	no addition Y, mg	with glucose Y, mg
glucose	1.0*	1.5	1.6*	-
fructose	1.3	1.3	1.6*	2.0
mannose	1.2	1.5	1.3	1.5
galactose	0.1	1.3	0.0	2.0*
maltose	0.4	1.2	0.3	1.1*
rhamnose	0.1	1.6	0.3	2.0
sucrose	0.2	1.5	0.0	2.2
ribose	0.1	1.3*	0.1	2.1
xylose	0.1	1.2*	0.1	2.1
standard medium	1.2	-	1.5	-

sources. Growth on glucose, fructose and mannose was good (Table 6). Sucrose, which is the carbon source of the M&S medium, turned out to be unsuitable. This was confirmed in another experiment in which also several carboxylates and Tween 80 were included. None of the additions allowed substantial growth; acetate, citrate, malonate and Tween 80 inhibited growth on glucose. Some growth on sucrose medium was found at the lower pH value of the M&S medium (4.2).

KNO_3, glutamate, aspartate, and urea, were compared with NH_4Cl. Nitrogen sources were added in concentrations equivalent to N contents (10.8 mM), 25 mM MES was used as pH buffer. Possible inhibitory effects were studied by addition of the standard concentration of NH_4Cl to the tested N sources. Growth on NH_4^+ was good, NO_3^- was unsuitable. Growth on aspartate, glutamate, and urea as single N-source was possible, but at least urea inhibited growth on NH_4Cl (Table 7).

Table 7. Effects of different nitrogen sources during 18 days (strain S1) and 20 days (strain T6) of incubation (* S.E. > 25%) at 20°C.

nitrogen source	strain S1		strain T6	
	no addition Y, mg	with NH_4Cl Y, mg	no addition Y, mg	with NH_4Cl Y, mg
none	-	-	0.6	-
NH_4Cl	0.9	1.1	1.9	1.8
KNO_3	0.0	1.1	0.6	2.0
aspartate	0.3	1.0	1.5	1.8*
glutamate	0.6	0.9	1.0	0.6*
urea	0.7	0.6	1.1	0.9
standard medium	0.9	-	1.7	-

A vitamin-free medium was tested and mycelia were subsequently transferred to a fresh vitamin-free medium or to the standard medium. Absence of vitamins did not prevent growth, not even upon several subcultures. However, growth on standard medium was better.

Emersed growth

Mycelial tufts, inoculated on pre-weighed filter paper bridges in liquid medium, grew better than submersed mycelia although the filter paper itself

Table 8. Yields of strains S1 and T6 grown emersed and submersed for 21 and 20 days respectively at 20°C.

	strain S1 Y, mg ± S.E	strain T6 Y, mg ± S.E.
emersed, on paper bridge	3.7 ± 1.0	3.5 ± 0.3
submersed, with paper	1.1 ± 0.3	1.0 ± 0.2
standard (submersed)	1.8 ± 0.1	1.3 ± 0.2

was slightly inhibitory to growth (Table 8). Growth on agar, covered with cellophane, resulted after 28 days in yields of about 20 mg per dish, i.e. 5 mg per tuft and per 5 ml. After 52 days, up to 30 mg dry weight was produced per dish, which amounts to 38% (w/w) of the amount of glucose available as a carbon source. Emersed mycelia grew more densely, rather than that they expanded more than submersed cultures.

Erratic growth of emersed tufts occurred more often than of submersed tufts. Therefore the influence of submersed versus emersed incubation on lag phase was tested separately. Tufts were inoculated onto F agar; for submersed incubation, agar squares of 3x3 cm were cut out and turned up side down. Microscopical observation showed that submersed tufts started to grow after 2 days, emersed tufts only after 4 days. Tufts on agar, lying in a drop of liquid medium, and tufts inoculated into a hollow in the agar showed intermediate lag periods. The lag phase was also shortened when emersed incubated tufts on F agar were covered with a coverslip.

Growth limitations

Unless mycelial growth is limited by some internal factor, restricted growth can be ascribed either to exhaustion of the medium or to accumulation of inhibitory substance(s).

To evaluate the growth potential of submersed colonies, 21 days old colonies were transferred to fresh F medium. Also new tufts were inoculated into a larger medium volume, 32 ml in 100 ml flasks, resulting in the same medium depth as 5 ml in tubes (14 mm). The data in table 9 show yields higher then standard values for Y_{max}; therefore mycelial growth is potentially unlimited.

To check exhaustion of nutrients, a half and a twice concentrated F medium were tested. Yields in half-concentrated medium were two thirds of

Table 9. Growth potential of submersed colonies in a larger medium volume or in refreshed medium (* initial dry weights: S1 1.3, T6 1.5 mg) at 20°C.

	strain S1		strain T6	
	day 20	day 28	day 20	day 28
	Y,mg± S.E	Y,mg± S.E.	Y,mg± S.E	Y,mg± S.E.
inoculum in 32 ml	1.5 ± 0.1	3.6 ± 0.2	1.5 ± 0.3	3.7 ± 1.0
transferred 21* days old colonies	2.4 ± 0.1	-	2.2 ± 0.2	-
standard (in 5 ml)	0.9 ± 0.3	1.5 ± 0.1	1.2 ± 0.1	1.5 ± 0.3

that in standard medium, growth in twice-concentrated medium was the same as in standard medium. Substrate exhaustion can therefore be ruled out.

Stale medium from 21 days old cultures was tested. After removal of the colonies it was brought to volume, filter-sterilized, and inoculated again. Stale medium was also used for emersed growth on pre-weighed filter paper bridges. No growth occurred. Stale medium diluted with water or fresh F medium (1:1; v/v) allowed growth of strain T6 to 0.3 mg, but prevented growth of S1. Stale medium adjusted to pH 5.5 and a glucose addition of 22.2 mM allowed growth of strain S1 to 0.2 mg and of strain T6 to 0.6 mg.

DISCUSSION

For optimal growth of submersed tufts, a new medium modification, a replacement of the tartrate buffer by succinate or MES is recommended. Although emersed incubation results in higher yields, because of the frequently erratic initial growth, experiments were continued with submersed cultures.

Growth curves

Both strains increased their growth rate from 1982 to 1984. This shift towards higher growth rates was already mentioned by Fries at delivery of his strain 740b (here: S1) in 1980. An increased growth rate potential as adaptation of cultures is not generally noted. However, sectoring of *Lactarius* cultures (Oort, 1981), and a general loss of symbiotic potential of

mycorrhizal fungi (Molina & Palmer, 1982) seem quite normal. Increased growth rates of plant calli by repeated subculturing is mentioned by Noguchi et al.(1977). Since mutagenic treatment decreased the required number of subcultures, this adaptation could possibly be based on a genotypical change.

The fitting of logistic functions does not satisfy completely. A steeper rise of the curves (indicating a higher specific growth rate (SGR)) and a sharper kink to the level of maximum yield would fit better in all cases. Logistic functions are meant to simulate growth of non-aggregated cells, the growth of which is ultimately terminated by nutrient exhaustion or by self inhibition. A filamentous fungus as C. cibarius is kept off exponential growth by the architecture of the colony form (Griffin, 1981); this probably causes aberration from the theoretically logistic growth.

Growth rates could not be improved by changing environmental conditions, apparently the specific growth rate of C. cibarius is a fundamental property which is difficult to manipulate. Maximum yield was almost always reached after 20 days. This means that differences in lag phase will be smoothened out at this time and are not reflected in ultimate dry weights.

General conditions

F medium turned out to be superior to other media. Filter sterilization is essential. The present study confirms that optimum growth is achieved around 22 ^{o}C and at a slightly acid pH.

The beneficial effect of succinate and MES as buffers may be connected with the smaller shift in pH enabling more growth at a favourable range of acidity. Differences in yields on succinate-buffered media with different initial pH values should be interpreted as effects of initial pH and not of pH shifts. On the one hand, these shifts are not large, on the other hand, optimum growth occurs at a slightly decreasing rather than at a constant pH. In this respect there is no objection against the use of the possibly acidifying ammonia ion as a nitrogen source. The critical pH value of 4.5 is possibly reached too quickly on tartrate-buffered medium. Effects of pH are generally considered to be complex because of differential uptake of cations and anions and because of secretion (Griffin, 1981). The observed rise in final pH values in succinate-buffered media with low initial pH remains unclear.

Chemically ill-defined additions to the F medium resulted in lower values for Y_{max}, and probably attributed to advanced medium staling. Presumed inert

medium carriers such as filter paper, sand, vermiculite, perlite, and even rayon fibre plugs proved to be inhibitory to growth (unpublished). Although the growth requirements of C. cibarius are basically simple (see below) apparently its growth is easily inhibited. The osmotic potential of the medium is not very critical; growth-reducing effects of salt additions cannot be ascribed to osmotic effects.

Nutrients

The apparent absence of absolute nutritional deficiencies, especially of vitamins and micro minerals, can be explained by nutrient transfer with inoculum tufts, or by chemical contamination of glassware or de-ionized water.

The carbon and nitrogen requirements of C. cibarius are similar to those of other mycorrhizal fungi, or have even more limited margins (Palmer & Hacskaylo, 1970; Molina & Palmer, 1982; Harley & Smith, 1983). At low pH, sucrose could be used as carbon source. This explains growth and also isolation success on M&S medium, which has an initial pH of 4.2 (Straatsma et al., 1985).

Emersed growth

Maximum emersed-growth yields were much higher than maximum yields of submersed mycelia. It is unclear whether the success of emersed incubation results from a higher specific growth rate or from a higher value for Y_{max} only. Emersed and submersed cultures of Cenococcum geophilum (graniforme), Mycelium Radicis atrovirens and Suillus (Boletus) variegatus showed equal maximum yields. Highest growth rates of these fungi were found for shaken submersed cultures, indicating a growth rate-limiting oxygen supply in standing cultures (Wikén et al., 1951; Wikén & Somm, 1952; Pedersen & Lindeberg, 1970). Oxygen diffusion may limit the growth rate but cannot itself explain a limited maximum yield.

Modess (1941) found that floating mycelia generally grew better than submersed ones. But slowly growing species (growth less than 1 cm per month) of the genera Lactarius and Russula grew on agar underneath the surface and probably preferred submersed incubation. L. flexuosus and Amanita rubescens could only be grown submersedly in liquid medium. This is also reported for the very slowly growing Elaphomyces granulatus (Jackson & Mason, 1984). C. cibarius is a very slowly growing species but it shows good emersed growth,

however, for initial growth it prefers submersed incubation.

Growth limitations

Submersed growth is limited by accumulation of inhibitory substances and not by nutrient exhaustion of the medium. Staling may result from accumulation of organic acids (Griffin, 1981), possibly also as an indirect result of oxygen deficiency. This could also be the case for submersed growth of C. cibarius; crystals were formed at elevated Ca levels and inhibition by staled medium was partly reduced after pH adjustment (and addition of glucose). Some carboxylates were found to be strongly inhibitory. Since early growth was not affected by ultimately growth-reducing substrates (yeast extract etc.), the production of staling compounds must have gradually increased. This phenomenon is discussed in general terms by Robinson (1969).

Higher yields of emersed incubated tufts can not be explained by an increased tolerance to staling substances, since emersed tufts on stale medium failed to grow. Therefore the production of staling substances must be lower under emerse conditions.

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INFLUENCE OF ROOTS ON GROWTH IN VITRO OF THE MYCORRHIZAL MUSHROOM CANTHARELLUS CIBARIUS Fr.

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ABSTRACT

The presence of living tomato roots was found to have a strong positive effect on in vitro growth of hyphal fragments of Cantharellus cibarius. This was observed for 12 of 14 strains tested.

Regeneration being the first phase of growth appeared to be independent of the presence of a root. For the actual development of strong mycelial growth 7 strains were found to be completely dependent on the presence of roots whereas 2 strains were independent. Five strains showed a considerably prolonged lag phase in the absence of roots. Analysis of growth curves indicated that the presence of roots had no influence on the specific growth rates.

Hyphal fragments of strain S1, which are totally root- -dependent for their growth, strongly grew in the absence of roots if they were brought into intimate mutual contact. Factor(s) stimulating growth seem therefore to be produced by both roots and fungus. The presence of the factor(s) could not be demonstrated in the culture medium or in dead root material.

ADDITIONAL KEYWORDS inoculum, lag phase, M-factor, root metabolite(s), specific growth rate

INTRODUCTION

In two previous papers on Cantharellus cibarius Fr., the isolation and axenic culture, and the nutritional requirements of mycelial cultures were described (Straatsma et al., 1985; Straatsma & Van Griensven, submitted). C. cibarius is considered to be an ectomycorrhizal fungus (Trappe, 1962; Froideveaux, 1975). Growth of these fungi is generally stimulated by the so-called M-factor from roots (Melin, 1954; Melin & Rama Das, 1954; Melin, 1963). M-factor activity was found in vitro by incubation of roots in complete nutrient media which, without roots, already support growth of many mycorrhizal fungi. This is quite distinct from in vivo activity of roots that supply, among others, carbohydrates to their symbiotic fungi (Melin & Nilsson, 1957; Harley & Smith, 1983). Chemical analysis of this M-factor has not been successful so far (Harley & Smith, 1983).

The specific growth rate (SGR) of C. cibarius in modified Fries medium was about 0.2 day^{-1} (a doubling time longer than 3 days), which is very low (Straatsma & Van Griensven, submitted). A higher SGR by M-factor activity would be important for cultivation and therefore for successful domestication of C. cibarius.

In this paper M-factor activity from living tomato roots on growth of hyphal suspensions of C.cibarius in modified Fries medium is described and evaluated by an analysis of growth curves. The factor(s) concerned turned out to be unspecific and even produced by the fungus itself.

MATERIALS AND METHODS

C. cibarius strains

Strains were taken from the culture collection of the Mushroom Experimental Station, one strain per origin (Straatsma et al., 1985). For detailed studies mainly strain S1, obtained by spore germination by N. Fries (his strain number 740 b) (Fries, 1979) was used. A working collection of strains was subcultured monthly and yearly replaced from the stock collection.

Root culture

A clone of excised tomato roots (strain Panase F; Pannevis, Enkhuizen,

the Netherlands) was grown as outlined by Butcher (1980). The medium of Murashige & Skoog (1962) was used, as by Montezuma de Carvalho & Guimares (1976) for potato roots. Sucrose was added at a concentration of 20 $g.l^{-1}$, and vitamins as by Meredith (1979). The medium was sterilized by autoclaving. Root sectors, segments of a main root axis bearing lateral roots, were weekly subcultured and served as an inoculum source for tip cultures. Floating, 1 cm long root tips cut from one week old sector cultures were transferred to 25 ml medium in 100 ml flasks and cultured in the dark at 27 °C. 7 days old tip cultures were used in experiments with C. cibarius.

Roots of Betula pubescens and Pinus sylvestris were cut from seedlings, pre-grown aseptically on Whatman No.1 filter paper strips with 10 ml medium of Krupa & Fries (1971) in glass tubes for 3 and 5 months respectively.

Cultivation of C. cibarius

Liquid, filter-sterilized, modified Fries (F) medium was used. For inoculum production submersed mycelia were cultured for 3 to 5 weeks in 10 ml medium in glass tubes (Straatsma & Van Griensven, submitted). Hyphal suspensions were prepared by shaking colonies with glass beads (6 mm diam.) in 50 ml medium by hand, leaving the core of the colonies intact (Wikén et al, 1951). The macerate was seaved through 1 mm nylon gauze and the filtrate was used as inoculum (Melin & Rama Das, 1954).
1 ml suspension, containing 10 to 50 µg dry weight, was pipetted into 25 ml fresh medium in 100 ml flasks, containing one pre-cultured tomato root. Flasks were closed with aluminium foil. Incidentally cultivation was done on 20 ml F agar (final concentration 12 $g.l^{-1}$) medium in petri dishes; dishes were sealed with strips of Parafilm. Cultures were placed in the dark at 20 °C. Experiments were done in 5 fold.

Experiments with separated roots

15 cm of dialysis tubing ('Visking' regenerated cellulose, pore size 2.4 nm, 25 mm width; Union Carbide Co., Chicago, U.S.A.) was tied at one end, washed in hot 0.1% EDTA (w/v), and sterilized by autoclaving. Tomato root tips were inoculated into tubings and pre-cultured.

Cellulose nitrate membrane filters (0.2 µm, Sartorius, Göttingen, F.R.G.) were glued with silicone glue to one end of a glass cylinder (30 mm diam.); 3 small glassbeads were glued to this, they served as support of the tubes. Tubes were washed in hot 0.1% EDTA (w/v) and sterilized by autoclaving.

Tomato roots were pre-grown in these tubes, or were transferred to the tubes after standard culture.

Tubing and tubes were placed in flasks in which a hyphal suspension of C. cibarius was inoculated. For membrane-glass tubes, 250 ml flasks with 50 ml of medium were used.

Yield determination

Cultures were poured into petri dishes and roots were carefully removed. The cultures were filtered with a vacuum funnel on washed and pre-weighed 1.2 µm cellulose nitrate filters (25 mm diam.)(low yields), or Whatman No.1 paper disks (25 mm diam.)(high yields), and rinsed twice with water. Filters with cultures were dried for 1 hour at 104°C and incubated overnight over silicagel. Weights were determined with an accuracy of 0.1 mg. Average yield and standard errors were calculated.

RESULTS

General growth

Without the presence of a root, both in liquid medium and on agar medium, hyphal fragments of C. cibarius strain S1 showed branching and development into small, very thin mycelia, as seen under a dissection microscope. No further development to strongly growing mycelia, such as from mycelial tufts for inoculum production, was found. The preliminary development into mycelia that were just visible to the naked eye is called regeneration. On agar medium the number of regenerating hyphae was higher when organic nutrients were omitted. This effect resulted from the absence of vitamins, particularly of biotin (Table 1). Hyphal fragments, incubated on agar medium without organic nutrients until maximal regeneration was reached, were transferred to liquid F medium but showed no development into normal mycelia.

In the presence of a living tomato root strong growth of strain S1 was observed after about three weeks of incubation; dense, submersed colonies grew attached to the added root. The root itself showed no continuing growth but apparently remained alive with a healthy, white tip. Later, also colonies not in contact with the root developed. As growth continued, colonies reached the medium surface and grew emersedly as well. Full-grown, 46 days old cultures, transferred to 250 ml of fresh medium in 1 l Fernbach flasks,

Table 1. Effects of omission of medium compounds on regeneration of hyphae of strain S1, after 31 days of incubation. (+: hardly any regeneration, ++: distinct regeneration, +++: optimum regeneration).

medium	measure of regeneration number of cultures		
	+++	++	+
minus organic components	5		
glucose		1	4
vitamins		5	
thiamin			5
pyridoxin			5
riboflavin			5
biotin		5	
nicotinamid			5
p-amino benzoic acid		1	4
pantothenic acid			5
inositol			5
control (complete medium)		1	4

continued to grow. They showed, within a week, development of many new colonies, presumably from originally inoculated hyphae hitherto in lag phase. In inoculum dilution experiments in the presence of roots, inocula as small as 0.2 µg were able to grow.

Growth in the presence of a detached birch or pine root was similar to growth with a tomato root. In further experiments only tomato roots were used, because they could be pre-cultured in a short time, resulting in homogeneous material.

No close histological interaction between C. cibarius and roots, i.e. mycorrhizal formation, occurred. Trials on mycorrhizal formation, using methods resembling those of Marx & Zak (1965), were done with intact seedlings of birch and pine. Autoclaved sand, vermiculite, and perlite, with or without peat moss, moistened with F medium, were used as substrates in 0.5 l or 2 l erlenmeyer flasks. Also filter paper strips, standing either in 5 ml F medium, or in the medium of Krupa & Fries (1971), were used in glass tubes. In all experiments growth of C. cibarius was unsuccessful, even if full-grown cultures were used as inoculum; mycorrhiza did not develop.

Growth curves

With developing mycelia repeated yield determinations in 5 fold were made. Logistic functions (equation 1 and 2) were fitted on measured dry weight data (Straatsma & Van Griensven, submitted).

$$Y_T = Y_{max} / (1 + C * e^{-SGR * T}) \quad \text{equation 1}$$

$$C = (Y_{max} / Y_i) - 1 \quad \text{equation 2}$$

(Y_T: yield on time T (mg), Y_{max}: maximum yield, C: constant, SGR: specific growth rate (day^{-1}), T: time (day), Y_i: initial yield, "effective inoculum")

Since lag phases could extend for a rather long period, inoculum dry weight (Y_0) was not considered in calculations. However, it served as an additional check to the validity of the fitted functions, because the calculated value for Y_i should be lower than the measured value for Y_0.

Dependent on the strains three types of growth were found with regard to curves of series with and without tomato roots. This is illustrated in Figure 1 by growth curves of strains S1, T6, and T3 (data after one year of subculture), as representatives of these types. Type 1 shows growth only in the presence of a root, type 2 shows also root-independent growth but with a prolonged lag phase, and type 3 shows root-independent growth. After early growth the root-independent strain T3 was inhibited by tomato roots (see Figure 1). Classification of strains according to these types is given in Table 2. Strains with root-independent growth occurring only after 150 days (T4 and T7) were classified in type 1. In experimental repeats strains S1 and T3 showed their characteristic growth type, T6 however, also showed growth of type 1. The difference between type 1 and type 2 growth apparently is not sharp. A slightly inhibitory effect of tomato roots was found for the root-independent strain T1, similar to strain T3.

Growth of the different strains varied also with regard to SGR (specific growth rate)(Table 3) and Y_{max} (maximum yield) (Table 4). In accordance with earlier results (Straatsma & Van Griensven, submitted) strain S1 showed an increasing SGR value in time. Highest Y_{max} values were found for strain S1,

Table 2. Classification of strains on dependence of the presence of a tomato root for strong growth of hyphae.

Type of growth	Strain numbers
1 (root-dependent)	S1 T4 T5 T7 T13 T16 T17
2 (temporarily dependent)	S2 T2 T6 T8 T15
3 (root-independent)	T1 T3

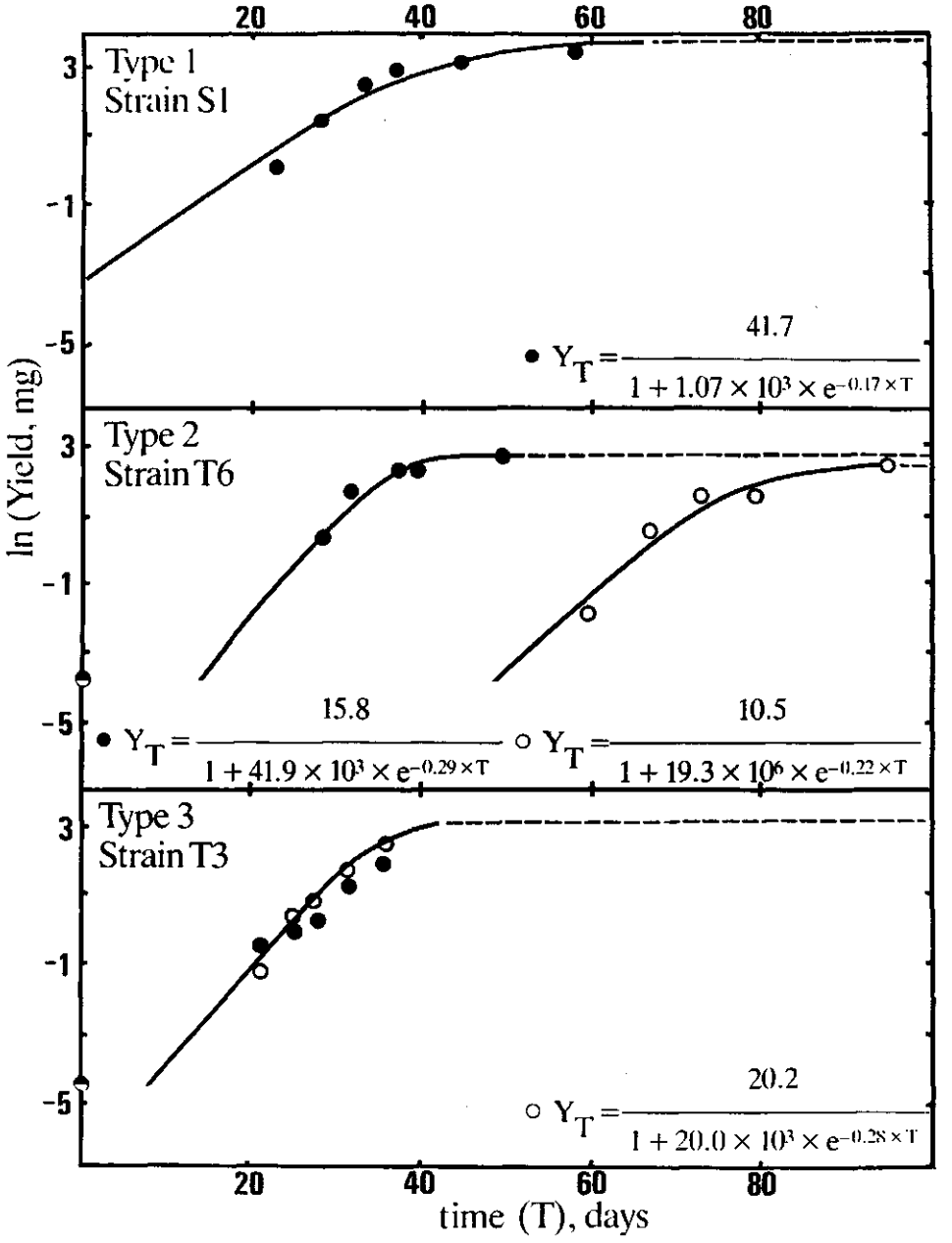

Figure 1. Fitted logistic functions of dry weight yields (Y_T) of three strains of C. cibarius in the presence or absence of a tomato root (solid and open symbols respectively). The value at T_0 is the inoculum dry weight.

Table 3. Classification of strains with regard to specific growth rate shortly after isolation.

Specific growth rate (day^{-1})	Strain numbers
< 0.20	S1 T3 T5 T8
0.20 - 0.25	S2 T1 T2 T7 T16
> 0.25	T4 T6 T13 T15 T17

Table 4. Classification of strains with regard to maximum yield in 25 ml medium.

Maximum yield (mg)	Strain numbers
< 15	T1 T4 T5 T8
15 - 25	T2 T3 T6 T7 T13 T15 T16 T17
> 25	S1 S2

40 mg per flask (25 ml) - a conversion of 40% of the available glucose -, corresponding with values found for emersed mycelial tufts (Straatsma & Van Griensven, submitted).

Although hyphal suspensions of strain S1 were completely dependent for strong growth on the presence of roots, its mycelial tufts can be grown without roots (Straatsma & Van Griensven, submitted). SGR values for hyphal suspensions co-cultured with roots were equal to values found for mycelial tufts (Straatsma & Van Griensven, submitted). An experiment with mycelial tufts cultured in the presence or absence of a tomato root confirmed this, in that no growth differences were found between treatments, not even with regard to duration of lag phase. It is clear that substances that are essential for growth of hyphal suspensions are produced by and fulfil requirements of successfully growing colonies of C. cibarius.

Root condition, separation from root, and root-independent growth

Pre-cultured roots exposed to either dipping in boiling water, incubation for 30 minutes at 50°C, incubation for 30 minutes at -20°C, or homogenization, failed to support growth. F medium containing root exudates, by pre-incubation of medium with a root (Melin, 1963), was inactive. Three weeks old, complete cultures that were killed and inoculated again (cf. Raveh et al., 1973) were inactive. Mycelium-conditioned medium, on which average yields of strain T3 were obtained varying from 1 to 10 mg per flask, did not support growth of a hyphal suspension of strain S1. Nutrients essential for

growth at low density of Vicia hajastana protoplasts (Kao & Michayluk, 1975), added to F medium, did not support growth.

No growth was found when a hyphal suspension was separated from a root by a dialysis membrane. Some growth was obtained when 0.2 µm filters were used as a separation membrane (Table 5).

To check the influence of inoculum density on growth, concentrated hyphal suspensions were tested. No growth occurred, not even when the inoculum dry weight surpassed that of mycelial tufts that showed normal growth (Table 6). In highly concentrated suspensions hyphae clumped together and occasionally showed growth. In a number of experiments, hyphae from suspensions were

Table 5. Effects of the presence of cellulose dialysis tubing, 0.2 µm pore membranes, and of separation of hyphae from tomato roots by these means on growth of strain S1 in 50 ml medium, after 55 days of incubation.

	Yield mg ± S.E.
standard (root present)	39 ± 18
control, with dialysis tubing	28 ± 5
control, with 0.2 µm membrane tube	38 ± 8
root in dialysis tubing	0
root in 0.2 µm membrane tube	4 ± 4

Table 6. Effect of inoculum condition on root-independent growth of strain S1 in comparison to that of hyphal suspensions of the root-independent strains T1 and T3 (compilation of different experiments, with different incubation periods).

	inoculum weight (µg)	strong growth
mycelial tuft S1	82	+
	71	+
	50	+
	22	+
hyphal suspension S1	65	-
	110	-
	150	-
hyphal suspension T1	22	+
hyphal suspension T3	10	+
	8	+

brought into intimate mutual contact by concentrating them by filtration on a limited area of a filter paper disk. They showed normal growth comparable to that of mycelial tufts if they remained attached to submersed incubated filter paper disks. Emersed incubation of such concentrated hyphal suspensions on filter paper disks on agar medium was unsuccessful.

DISCUSSION

The effect of the presence of living roots on the growth of hyphal fragments was very strong for most of the tested strains of C. cibarius. However, neither hyphal regeneration as such, nor the specific growth rates (SGR) were affected by the roots. Type 2 growth (Fig. 1) demonstrates that the presence of roots only shortens the duration of the lag phase of growth. Type 1 growth should also be explained in such a way. The presence of roots shortens an otherwise continuous lag phase. A potential for successful, root-independent growth of hyphal suspensions is evident; hyphal suspensions of some strains were root-independent, as were mycelial tufts and also hyphae brought into intimate mutual contact of the dependent strain S1. Also remarkable was the luxuriant growth of not yet growing hyphae when full-grown cultures were transferred to 250 ml of fresh medium; this indicates a stimulation by the already established mycelia. The discrepancy between unsuccessful growth of hyphal suspensions and successful growth of mycelial tufts could be explained by mutual stimulation of hyphae in a mycelial tuft.

Strain T5 showed reluctant growth, i.e. a combination of low SGR and low Y_{max} (maximum yield) values, even in the presence of roots. Next to a potentially low SGR value or lack of unknown nutrients, sensitivity to submersed incubation and self-produced inhibitors (Straatsma & Van Griensven, submitted), or to inhibition by root products (Melin, 1963; this study) might explain this.

Inoculum

The positive effect of roots has only to be considered with regard to the kind of the inoculum applied, i.e. hyphae (hyphal fragments); mycelial tufts were not affected. The effect of roots on the establishment of axenic cultures of C. cibarius was quite similar; spore germination required a living stimulator, but fruitbody tissue explants could be cultured without

this (Straatsma et al., 1985). Negative experiences with growth of small inocula of mycorrhizal fungi were mentioned by Oort (1974) for Lactarius species and by Fries (1978) for Amanita muscaria. More detailed results with hyphal suspensions were published by Wikén et al. (1951) for two mycorrhizal fungi, and by Dijkstra et al. (1972) for Agaricus bisporus; both studies show better (earlier) growth at higher densities. Similar results are well known for plant cell suspension cultures (Kao & Michayluk, 1975) as well as for animal cells (Paul, 1975).

Nature of the root factor

The root factor is of a very general nature, nevertheless its chemical nature remains obscure. In contrast to results of Melin (1963), root exudates and root skeletons proved to be inactive in the present study. A continuous supply of (root) metabolite(s) seems essential, these metabolite(s) apparently being transient. Stimulatory substance(s) were slightly able to pass 0.2 um pored filters, but not dialysis tubing. The requirement for (initial) contact was also found for hyphae when brought into intimate mutual contact. Macromolecular and/or cell-wall bound substances might be important. Further experiments on growth of hyphal suspensions of C. cibarius are necessary to elucidate the nature of stimulation.

Melin and Rama Das (1954) concluded for Russula xerampelina and Boletus (Suillus) variegatus that they were 'totally deficient', respectively 'partial(ly) deficient' for metabolites from pine or tomato roots. The latter species was 'capable of synthesizing the factor concerned, but not as rapidly as needed for optimal growth'. This opinion implicates an increased SGR value by M-factor activity. Growth curves (also Melin, 1963), however, do not prove this explicitely. R. xerampelina depended strongly on root metabolite(s) since it was routinely cultivated in the presence of tomato roots, nevertheless some root-independent growth occurred: 'in the controls the hyphal growth was very poor and soon stopped completely'. Hyphal regeneration of C. cibarius, strain S1, on agar medium resembles this very much. Benedict et al. (1965) used floating mycelial tufts to study the influence of living tomato roots. Only 1 out of 8 tested species was stimulated by roots, 3 species were more or less inhibited. Very slowly growing cultures of 5 species, refractory to normal cultivation, were obtained when roots or their exudates were applied. Many species could not be cultivated, not even with roots. Since Melin and Rama Das (1954) and Melin (1963) also reported

inhibitory root effects, Benedict et al. (1965) concluded to pronounced inhibitory effects in their experiments and not to a general, root-independent growth. Nylund & Unestam (1982) do not agree with a general nature of the M-factor; they found growth stimulation of mycelial tufts of Piloderma croceum, but not of 7 non-mycorrhizal fungal species, by Picea abies roots. However, Suillus bovinus was not stimulated by roots and P. croceum was also stimulated by germinating seeds.

The general nature of the M-factor is furthermore stressed by effects of non-host plant roots (Melin & Rama Das 1954; Benedict et al., 1965; this study), and also by root effects on non-mycorrhizal fungi; i.e. on Agaricus augustus (Benedict et al., 1965), on Aspergillus, Penicillium, and Stycanus species (Shemakhanova, cited in Harley & Smith (1983)), and on Agaricus bisporus (G.M. Jansen, unpublished).

Independence of host roots is not only shown for vegetative growth, but also for formation of a mycorrhizal mantle on artificial 'roots' of silicone by Boletus (Suillus) variegatus (Read & Armstrong, 1972), and for formation of primordia and/or small fruitbodies of several mycorrhizal Boletus s.l. species (Giltrap, 1981).

Quite different are effects of host roots on spore germination of particular mycorrhizal fungi (Fries, 1982), or on recognition phenomena in the natural establishment of mycorrhiza by particular symbionts (Harley & Smith, 1983). The negative results with mycorrhizal synthesis experiments between C. cibarius and tree seedlings, caused by total inhibition of mycelial growth, prevented a study of a more natural influence of roots. Definite conclusions about the M-factor can only be made after elucidation of the delicate requirements of hyphal suspensions.

Other biotrophic fungi

Striking parallels to our results are found with other biotrophic fungi from various taxonomical and ecological groups. Growth of Endogonaceous fungi from vesicular-arbuscular mycorrhiza is possible in dual cultures with roots (Mosse & Hepper, 1975). Some growth without roots, or spores - which contain important nutrients -, is reported by Hepper (1983); unlimited growth does not occur. The downy mildew of pearl millet, Sclerospora graminicola, can be cultured on callus, also of non-host origin, and survival on callus-conditioned medium occurs (Bhat et al., 1980); however, independent growth is not yet reported. The white-pine blister rust, Cronartium ribicola,

can be grown on a suitable medium (Harvey & Grasham, 1974) but it was quite refractory to plant callus-independent growth in earlier attempts. Physical contact with callus was not essential and growth with non-host callus occurred (Harvey & Grasham, 1971). Survival on callus-conditioned medium was possible (Harvey & Grasham, 1970), which can be explained as a sustaining lag phase of growth. Effects of inoculum density and mutual growth stimulation of germinating spores of Puccinia species were reported by Kuhl et al. (1971).

Harvey & Grasham (1974) stated that 'nutrition as such' is not 'the determinant of the obligate nature and host selectivity', a view comparable to that of Harley & Smith (1983) that 'little has been discovered which differentiates ectomycorrhizal fungi from other fungi in their physiology' by growth experiments; this view is supported by the present study.

In conclusion we find, as opposed to Melin (1963), M-factor activity strictly confined to living root material. This material does not affect the specific growth rate of the fungus, but only reduces or abolishes the lag phase. The ecological implication is that the establishment of the fungus requires the presence of living roots, while, once established, the organism can maintain its own development. The distinction between symbiotic and saprofytic fungi, if existing at all, is the requirement not of a specific root factor (Melin, 1963), but of a general product of plant metabolism.

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CARBON DIOXIDE AS A GROWTH FACTOR OF HYPHAE OF THE MYCORRHIZAL MUSHROOM CANTHARELLUS CIBARIUS Fr.

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ABSTRACT

Volatile(s) from donor cultures of tomato roots, Cantharellus cibarius, Boletus edulis, and Tylopilus felleus stimulated the development of mycelia in receiver cultures of hyphal fragments of C. cibarius. This effect was prevented by the presence of a KOH solution. Donor cultures could be replaced by 0.5% CO_2, but the existence of additional factor(s) was indicated.

In contrast to the further development into a mycelium, the initial outgrowth of hyphal fragments was not affected by CO_2.

ADDITIONAL KEYWORDS donor-receiver cultures, M-factor, volatile(s)

INTRODUCTION

In a previous paper the effect of living roots from different species on hyphal growth of Cantharellus cibarius Fr. was described (Straatsma & Bruinsma, submitted). Initial growth of hyphal fragments (regeneration) was not affected, and mycelial tufts grew independently of the presence of a root. However, the development of regenerated hyphae into a mycelium required the presence of a living root in 12 out of 14 strains. The positive effect was limited to a shortening of the lag phase, the specific growth rates and

maximum yields being unaffected. Apparently, the metabolite(s) involved were general in nature and were produced by both the plant root and the fungus. The metabolite(s) could not be detected in the medium apart from the living root. Further elucidation of this factor might enable homogeneous growth of hyphal suspensions and probably also successful cultivation of germinated spores, that have to pass the stage of a small hypha.

Since the metabolite(s) are transient, their nature may be volatile. Different organisms were tested as donors of volatile metabolite(s) that may affect hyphal growth. The very sensitive strain S1 was compared with the less sensitive strain T6 and the independent strain T3. The effects were analysed in a replacement study. Carbon dioxide concentrations above the ambient atmospheric level turned out to be required for the development into mycelia.

MATERIALS AND METHODS

Organisms

Three strains of C. cibarius, S1 (= strain 740b of N. Fries), T3, and T6, were tested. These strains were chosen because their hyphal suspensions reacted differently to root metabolite(s) (Straatsma & Bruinsma, submitted); S1 was totally dependent on the presence of roots, T3 independent, and T6 had an intermediate position.

The following organisms were used as possible donors of the metabolite(s):

- tomato roots, commercial strain Panase F (Pannevis, Enkhuizen, the Netherlands)
- C. cibarius, the above mentioned strains
- Boletus aestivalis, strain AE1, isolated from tissue of a fruitbody collected at Horst, the Netherlands
- Tylopilus (Boletus) felleus, strain FE1, isolated from tissue of a fruitbody collected at Horst, the Netherlands

Tomato roots were subcultured once a week as root sectors (Butcher, 1980; Straatsma & Bruinsma, submitted). All fungi were taken from a working collection that was subcultured monthly. This collection was replaced once a year from the stock collection of the Mushroom Experimental Station (Straatsma et al., 1985) which also contains the bolete species.

Cultivation

Tomato roots were grown on 20 ml autoclaved M&S medium (Murashige & Skoog, 1962; Straatsma & Bruinsma, submitted), solidified with 12 $g.l^{-1}$ agar, in 9 cm petri dishes. To obtain superficial growth, necessary for optimal release of volatile substances, the medium was covered with filter paper (Whatman No. 1) on which a root tip was inoculated. The dishes were sealed with Parafilm and incubated for 8 days at 27°C in the dark.

Fungal cultivation was done with filter-sterilized, modified Fries (F) medium. Inoculum was always cultivated as submersed mycelium in 10 ml liquid F medium in glass tubes (Straatsma & Van Griensven, submitted).

To obtain fungal donors, 20 ml F medium, solidified with autoclaved agar to a final concentration of 1.2% (w/v), in 9 cm petri-dishes, were inoculated with 3 mycelial tufts. The dishes were sealed with strips of Parafilm and incubated for about 3 weeks at 20°C in darkness.

Hyphal suspensions of C. cibarius were prepared by shaking submersed mycelium cultures with glass beads in medium and filtering through 1 mm nylon gauze (Wikén et al., 1951; Straatsma & Bruinsma, submitted). Plates with F agar medium were inoculated with 0.5 ml of this suspension (containing 10 to 50 µg dry weight). The suspension was spread over the surface with a glass rod. To ease manipulation with these receiver dishes, hyphal fragments were stuck onto the agar surface by opening the dishes in the sterile air stream of a laminar airflow unit for 30 min. In order to compare these experiments on agar medium with the various ones in liquid medium (Straatsma & Bruinsma, submitted) control experiments were done in 100 ml flasks, holding 25 ml liquid medium, capped with aluminium foil.

Donor-receiver experiments

One donor dish was combined with one receiver dish, the latter was inverted and served as a lid of the first. Paired dishes were sealed with a double strip of Parafilm.

In some experiments the effect was tested of the presence of 1 M KOH or KCl solutions or water in donor-receiver combinations. A small porcelain cup with the test liquid was placed in the donor dish where a corresponding part of the agar medium was removed. The cup contained 4 ml of test liquid and also a folded filter paper strip to increase the area of the liquid.

Carbon dioxide supply

Three methods were used:

- Gas-tight 2.5 or 7.5 l Gaspak System containers (Becton Dickinson, Cockeysville, U.S.A.) were used for incubation of petri dishes. Carbon dioxide was generated with Na_2CO_3 and 5% (w/v) HCl, which were kept separately in a two-compartment dish until the containers were closed. To reach a 0.5 % (v/v) concentration of carbon dioxide, 0.56 and 1.68 mM Na_2CO_3 were required for the 2.5 and the 7.5 l containers respectively.
- 100 l polyethylene containers (Emergo, Landsmeer, the Netherlands) were continuously flushed with CO_2 / air mixtures (0.03 - 2.0 % (v/v)) from calibrated gas cylinders (Hoekloos, Schiedam, the Netherlands). Containers were closed with glass plates that were greased to the brim of the container with vaseline. For a build up of the desired concentration, the containers were flushed with 8 $l.hr^{-1}$ for the first 4 days, thereafter with 4 $l.hr^{-1}$. The actual concentrations were not checked any further, but a slight overpressure in the containers prevented penetration of the ambient atmosphere.
- An automatic CO_2 incubator (Precision, G.C.A. Corporation, Chicago, U.S.A.) with internal air circulation and operating at about 0.5% CO_2.

Petri dishes were incubated without sealing; flasks were loosely capped.

Yield determination

The contents of the receiver dishes were transferred to 150 ml beakers with 60 ml water, autoclaved at 120 °C and quickly cooled to 100 °C to redissolve agar. Cultures were filtered on pre-weighed filter paper disks (Whatman No. 1) and washed twice with hot water. Cultures in liquid medium were harvested without boiling treatment. Disks with cultures were dried for at least 1 hour at 104 °C and incubated overnight over silicagel. Their dry weights were determined with an accuracy of 0.1 mg. Average yield and standard errors were calculated per treatment.

RESULTS

Donor-receiver experiments

Volatiles from donor cultures successfully stimulated receiver cultures of strains S1 and T6 (Tables 1 and 2). Strain T3 grew also without being

paired to a donor culture, as was expected by its root-independent growth (Straatsma & Bruinsma, submitted). The general nature of the required factor(s) is stressed once more because all donor species tested were stimulatory (Tables 1 to 3). In an experiment with spores it was found that volatile(s) from a tomato root donor stimulated germination of spores as was found previously in co-culture experiments (Straatsma et al., 1985).

The presence of 1M KOH in paired dishes prevented growth of hyphae (Table 3). Control experiments with water and a KCl solution showed that the KOH effect was neither due to the presence of a container as such, nor to an

Table 1. Influence of volatiles from donor cultures on receiver cultures of hyphae of strain S1. Yields determined after 35 days.

Donor culture	Yield (Y) Y,mg ± S.E.(n)
tomato roots	9.1 ± 5.9 (7)
C. cibarius, S1	6.6 ± 4.0 (5)
none	0.2 ± 0.1 (5)

Table 2. Influence of volatiles from donor cultures on receiver cultures of hyphae of strains T6 and T3. Yields determined after 35 days of incubation.

Donor strain (*C. cibarius*)	Receiver strains T6 Y,mg ± S.E.(n)	 T3 Y,mg ± S.E.(n)
Strain S1	22.9 ± 5.1 (4)	7.7 ± 2.8 (5)
Strain T6	13.8 ± 7.6 (5)	6.1 ± 2.6 (5)
Strain T3	10.8 ± 6.7 (5)	9.0 ± 3.5 (5)
none	0.4 ± 0.1 (5)	6.8 ± 1.3 (5)

Table 3. Influence of the presence of a 1 M KOH solution on the activity of volatiles from donor cultures on receiver cultures of hyphae of strain S1. Yields determined after 33 days.

Donor culture	KOH absent Y,mg ± S.E.(n)	KOH present Y,mg ± S.E.(n)
C. cibarius S1	22.5 ± 1.2 (3)	0.0 ± 0.0 (2)
B. aestivalis	23.5 ± 2.8 (3)	0.2 ± 0.1 (2)
T. felleus	23.8 ± 1.5 (3)	0.1 ± 0.0 (2)
none	0.5 ± 0.2 (5)	–

effect on the water potential of the atmosphere (Table 4). Since the factor was absorbed by a KOH solution further attention was focussed on effects of carbon dioxide.

CO_2 effects

Preliminary experiments on replacement of donor cultures by CO_2 addition were done in the Gaspak System containers. The atmosphere in the containers was refreshed once a week. No attention was paid to a possible decline of the CO_2 concentration during a week. The experiments showed that CO_2, added at a concentration of about 0.5% (v/v), enabled growth of hyphae of strain S1 but C. cibarius donor dishes were more effective than CO_2 (Table 5).

Different CO_2 concentrations in air from gass cylinders were also tested. Strains S1 and T6 were used, S1 was also inoculated in liquid medium. Both strains reacted positively to CO_2; strain T6 was already affected by 0.08% CO_2, strain S1 by 0.22%. It was thought that gass diffusion to liquid medium in erlenmeyer flasks was not optimal, however, good growth was obtained (Table 6).

These experiments demonstrate that CO_2 is essential for the development of hyphae into mycelia. Other volatiles from donor cultures, or perhaps

Table 4. Effect of different solutions on stimulation of strain S1 by volatiles from donor cultures of strain S1. Yields determined after 31 days of incubation.

Solution in test atmosphere	Y, mg ± S.E.(n)
1M KOH	0.3 ± 0.1 (4)
1M KCl	10.0 ± 3.2 (5)
water	11.0 ± 1.8 (5)
none	7.5 ± 1.7 (4)

Table 5. Effects of volatiles from donor cultures of C. cibarius, S1, and of 0.5% CO_2 on receiver cultures of hyphae of C. cibarius, S1.

Incubation period (days)	donor culture	carbon dioxide
	Y, mg ± S.E.(n)	Y, mg ± S.E.(n)
30	1.9 ± 1.0 (3)	0.6 ± 0.2 (5)
36	7.8 ± 5.1 (4)	3.0 ± 1.4 (5)

non-volatiles might play an additional role. As already mentioned, comparison between CO_2 addition and incubation with a donor culture resulted in higher yields of the latter. A similar result was found when co-culture with roots (according to Straatsma & Bruinsma, submitted) was compared with CO_2 addition in a CO_2 incubator, both in liquid medium; roots resulted in earlier growth (Figure 1). The Y_{max} (maximum yield) value (8.9 mg / 25 ml), is similar to values found for submersed mycelial tufts (about 2 mg / 5 ml) (Straatsma & Van Griensven, submitted). For a third experiment on factors additional to CO_2, medium was used to culture strain S1 until a yield of 1.5 mg per 25 ml was obtained. It was then filter-sterilized, re-inoculated, and incubated in a CO_2 incubator. It resulted after 29 days of incubation in 4.8 $\pm$ 0.7 (5) mg, compared to 2.3 $\pm$ 0.4 (4) mg in standard medium. The acidity of the medium was not affected by incubation in 0.5% CO_2; therefore a pH effect can be ruled out.

The metabolic fate of CO_2 was studied by adding carboxylation products (Griffin, 1981) to F medium at 1 mM concentrations. Malic acid or combinations of organic acids including malic acid, were sometimes stimulatory, but reproducibility of these results was erratic. On agar medium irregular results were sometimes obtained with CO_2 incubation. However, this was always due to erratic first development of hyphal fragments (regeneration) and not because further development into a strongly growing mycelium was prevented. In liquid medium such an erratic start was not observed. Erratic regeneration seemed to occur more often at the end of these studies, after 5 years of subculture of strain S1; it was not noted in some general experiments on agar medium previously published (Straatsma & Bruinsma, submitted).

Hyphal regeneration

Omission of organic substances from the F medium (Straatsma & Bruinsma, submitted) and initial covering of inoculum on agar medium resembling submersed incubation, (Straatsma & van Griensven, submitted) were found to have positive effects. Both treatments were tested in the presence of 0.5% CO_2. Omission of glucose and vitamins resulted in a higher frequency of hyphal regeneration compared to regeneration on the standard medium. Omission of vitamins, addition of 1 mM K-malate, and incubation without CO_2 resulted in a yield of 18 $\pm$ 2 mg (5) after 90 days; growth on standard medium and on (complete) medium with K-malate was negligible. A hyphal suspension on F agar

Table 6. Influence of different CO_2 concentrations on growth of strains S1 and T6 after 33 days of incubation.

% CO_2 (v/v)	Strain S1		Strain T6
	liquid medium	agar medium	agar medium
	Y,mg ± S.E.(n)	Y,mg ± S.E.(n)	Y,mg ± S.E.(n)
0.036	0.0 ± 0.1 (10)	0.3 ± 0.1 (5)	0.6 ± 0.1 (8)
0.083	0.1 ± 0.7 (10)	0.8 ± 0.1 (5)	4.8 ± 0.9 (6)
0.22	8.9 ± 0.9 (10)	4.3 ± 2.2 (5)	21.2 ± 2.7 (8)
0.64	5.7 ± 3.8 (10)	10.9 ± 2.3 (6)	19.3 ± 2.2 (9)
2.03	9.4 ± 1.7 (10)	11.5 ± 4.4 (6)	24.4 ± 1.4 (8)

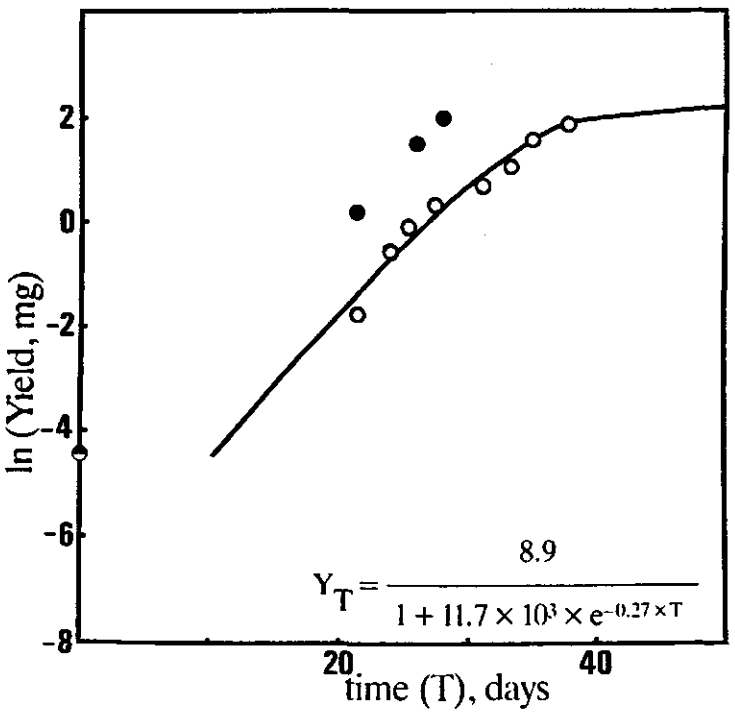

Figure 1. Fitted logistic function on dry weight yields (Y) of cultivated hyphal fragments under CO_2 incubation (Maximum Yield = 8.9 mg; Specific Growth Rate = 0.27 day^{-1}). Solid symbols: control series with living tomato roots. The value at day 0 is the inoculum weight.

medium was covered with a 1 mm thick silicone sheet (Rubber, Hilversum, the Netherlands), which is permeable to CO_2. As seen under a dissection microscope, the frequency of hyphal regeneration, especially of small fragments, was much better than in the uncovered control.

DISCUSSION

In this study it is shown that the growth stimulation of C. cibarius hyphae by living tomato roots, as well as mutual stimulation of hyphae (Straatsma & Bruinsma, submitted), are based on the production of volatile(s), which could be largely replaced by CO_2. This implies that M-factor activity from roots (Melin, 1963) in the case of C. cibarius is based on CO_2 production. It explains why dead root material and extracts are ineffective (Straatsma & Bruinsma, submitted).

CO_2 saturation concentrations for development into mycelium are strain-dependent. A concentration of 0.5% CO_2 (v/v) was sufficient for the most sensitive strain, S1. T3 grew also under ambient conditions, similar to its root-independent growth (Straatsma & Bruinsma, submitted). T3 was not tested under CO_2-free conditions. Strain T6 had a lower saturation value than strain S1, in accordance with its intermediate requirement for root metabolite(s) (Straatsma & Bruinsma, submitted).

In a previous paper (Straatsma & Bruinsma, submitted) it was described that without a root hyphal fragments only showed successful growth when they were brought into intimate mutual contact as in a mycelial tuft; single hyphal fragments showed regeneration but no development into strongly growing mycelia. CO_2 produced by respiration will dissipate into the environment unless it can accumulate locally between hyphae in a mycelium of a certain size and/or density. This explains both the growth of a mycelial tuft and the continuing lag phase of a hyphal suspension of the same inoculum weight, as well as the growth of hyphae brought into intimate mutual contact (Straatsma & Bruinsma, submitted).

Control experiments in which growth stimulation by CO_2 was compared with stimulation by volatiles from a C. cibarius culture, direct co-culture with roots, or use of mycelium conditioned medium, showed that although CO_2 is a key factor to overcome the lag phase of growth, (an) additional factor(s) exist(s).

Griffin (1981) states that CO_2 is 'probably a universal requirement for fungal growth', but that this requirement is 'somewhat difficult to ascertain because of the rapid production during respiration', and that particularly spore germination studies can confront with this requirement. In an early report Durrell (1924) showed that stimulation of spore germination of Basisporium gallarum (Syn. Nigrospora oryzae) by plant tissue was based on the presence of CO_2; saturation was reached at 1%. More recently it was shown that colony initiation in the flax rust, Melampsora lini, depended on self-produced volatiles. Again CO_2 was the active substance, its saturation concentration was below 1% (Boasson & Shaw, 1979, 1980). Only the establishment of colonies and not the germination of spores as such was affected (Boasson & Shaw, 1984). Another interesting example is the requirement for CO_2 of hyphae of the cultivated mushroom, Agaricus bisporus; its saturation value is very low (about 0.1%), which in fact required experiments with removal of CO_2 by KOH. Above a certain inoculum size (about 50 cells per fragment) endogenously produced CO_2 was sufficient (San Antonio & Thomas, 1972). Lösel (1964) reported that although spore germination of A. bisporus requires the presence of a volatile this is not CO_2. The difference between spore germination and hyphal growth found for M. lini and A. bisporus probably also exists for the mycorrhizal fungus Leccinum aurantiacum. Extensive research on spore germination by Bjurman & Fries (1984) showed that production of germ vesicles or germ tubes required no CO_2 or general nutrient, but a specific factor which was isolated and concentrated. It would be interesting to study the requirements for further development of these germinated spores. Spore germination of C. cibarius could well be analogous. Germination was stimulated by co-culture with living tomato roots, volatiles emitted from the fungus Ceratocystis fagacearum (Straatsma et al., 1985), and by volatiles from tomato root donor cultures (this study). This suggests a positive role of CO_2, which is certainly required for development of germinated spores (resembling small hyphae) into mycelia. Spore-germination frequency remained low, probably because a specific germination factor was lacking.

Possibly inhibitory effects of higher CO_2 concentrations were not studied because many fungi are very tolerant in this respect (Tabak & Cooke, 1968). However, 5% CO_2 was found to reduce hyphal growth from germinated spores of the vesicular-arbuscular mycorrhizal fungus Glomus mosseae (Le Tacon et al., 1983).

In the present study the metabolic fate of the CO_2 has not yet been thoroughly investigated. Preliminary experiments indicate that carboxylation into malate is not the main pathway.

It is concluded that hyphae of C. cibarius require enhanced concentrations of carbon di oxide for the development of mycelia. In nature living roots can contribute to satisfy this CO_2 requirement that enables the fungus to enter a mycorrhizal symbiosis. Once established, the mycelia can maintain their growth by their own production of respiratory carbon dioxide.

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CARBOXYLATED METABOLIC INTERMEDIATES AS NUTRITIONAL FACTORS IN VEGETATIVE GROWTH OF THE MYCORRHIZAL MUSHROOM CANTHARELLUS CIBARIUS FR.

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SUMMARY

Hyphal fragments of C. cibarius grew strongly in a nutrient solution supplemented with malic acid, thymine, and Tween 80. The mixture of these three substances replaced CO_2 or living roots which were used in earlier studies. Out of several possible CO_2 fixation reactions, those involved in the replenishment of Krebs cycle intermediates, in the synthesis of pyrimidines, and in the synthesis of fatty acids are growth limiting for hyphal fragments.

ADDITIONAL KEY WORDS: carbon dioxide, anaplerotic pathway, pyrimidines, fatty acids.

INTRODUCTION

In an earlier paper, the effect of living roots on the growth of hyphal fragments of Cantharellus cibarius Fr. was described. The positive effect was limited to a shortening of the lag phase, the specific growth rates and maximum yields of mycelial dry weights being unaffected. In contrast to

hyphal fragments, mycelial tufts grew independently of the presence of roots. It was concluded that the metabolite(s) involved were of a general nature and were produced by both the plant root and the fungus (Straatsma & Bruinsma, submitted). In a subsequent paper it was described that volatiles emitted by roots or mycelia could reduce the length of the lag phase of growth of the hyphal fragments. The volatile substance(s) could be replaced by carbon dioxide. A 0.5 % CO_2 concentration in the incubation atmosphere was sufficient for successful growth. Yields, determined after up to 35 days, indicated a reasonably short lag phase. However, the length of the lag phase varied between different experiments under identical conditions (Straatsma et al., submitted).

Both growth and morphogenesis of fungi are affected by CO_2 (Tabak & Cooke, 1968; Griffin, 1981). The incorporation of CO_2 into cell constituents of fungi is clearly shown by feeding experiments with radioactively labeled CO_2 (Foster et al., 1941; Gitterman & Knight, 1952; Buston et al., 1966; Hartman et al., 1972; Boasson & Shaw, 1984). Labeled CO_2 is incorporated in many low- and high-molecular weight substances. According to Zubay (1983) and Lehninger (1976) heterotrophic carboxylation reactions occur in intermediary metabolism in the synthesis of

- oxaloacetic acid and/or malic acid from phosphoenolpyruvate and/or pyruvate; these are anaplerotic routes for support of the Krebs cycle (Casselton, 1976)
- carbamoyl phosphate from glutamine or NH_4^+ as intermediary products in arginine and pyrimidine syntheses
- 5'-phosphoribosyl-5-aminoimidazole-4-carboxylate from 5'-phosphoribosyl--5-aminoimidazole in purine synthesis
- malonyl-CoA from acetyl-CoA in fatty acid synthesis
- β-methylglutaconyl-CoA from β-methylcrotonyl-CoA in leucine degradation and possibly involved in isoprenoid synthesis
- methylmalonyl-CoA from propionyl-CoA in odd-fatty acid degradation.

In fixation experiments with labeled CO_2, pyrimidines could probably receive a label indirectly by incorporation of aspartate. Direct labeling of fatty acids seems impossible because the fixed carbon atom is released again in subsequent condensation reactions (Zubay, 1983). By reversibility of reactions in the Krebs cycle and in glycolysis labeled compounds from anaplerotic CO_2 fixation could spread into all metabolic pathways. In our

view such experiments with gaseous, labeled CO_2 will hardly lead to interpretable results. Therefore we have chosen in our nutritional experiments with hyphal fragments of C.cibarius, for the replacement of CO_2 by carboxylated reaction products in order to reveal the metabolic role of CO_2. Moreover such a replacement will be useful in axenic experiments in which homogeneous growth of fragmented hyphae is required, for example in studies on fruitbody formation.

MATERIALS AND METHODS

The materials and methods used are similar to those of Straatsma & Bruinsma (submitted) and Straatsma et al. (submitted).

Two strains of C. cibarius, S1 (= polysporous strain 740b of N. Fries) and T6 (own isolation from fruitbody tissue, Straatsma et al., 1985), were tested. Cultivation was done submersed in liquid, filter-sterilized, modified Fries (F) medium (Straatsma & Van Griensven, submitted). Incubation was done in the dark at 20^{o}.

For inoculum production, the strains were cultivated in glass tubes as submersed mycelium in 10 ml medium. The inoculum used in the growth experiments consisted of a suspension of hyphal fragments. Such a suspension was prepared by shaking mycelium cultures with glass beads in medium. The resulting coarse suspension was filtered through 1 mm nylon gauze, and 1 ml aliquots were used as inoculum.

The replacement experiments were done under ambient atmospheric CO_2 levels. Hyphal fragments were cultured in 100 ml erlenmeyer flasks, holding 20 ml nutrient solution. Various intermediary products of CO_2 fixation were tested in F medium. In addition, some related substances were also investigated. The tested substances were:

- oxaloacetate and malate, in addition also fumarate, succinate, glutamate, and aspartate
- arginine, orotic acid, cytosine, thymine, and uracil
- guanine, adenine, and hypoxantine
- malonic acid and, in addition, Tween 80 (a mono-oleate of polyoxyethylene-(20)sorbitan)
- mevalonic acid

Methylmalonyl-CoA degradation results in succinic acid formation; succinic acid effects were considered with regard to anaplerotic routes.

In preliminary experiments, products involved in anaplerotic routes were used at a concentration of 2.8 mM, equivalent to the amount of gaseous CO_2 applied in our earlier experiments. Later, 1 mM concentrations were used. Pyrimidines and purines were tested at a concentration of 0.1 mM, according to Diner & Mott (1982). Tween 80 was tested because it can be used successfully as a fatty acid source for growth of the actinomycete Frankia spec. (Blom et al., 1980). The acidity of the media was adjusted to pH 5.5 with KOH or HCl solutions.

A control experiment was done in which mycelial tufts were used as an inoculum (according to Straatsma & Van Griensven, submitted).

Experiments were finished with dry weight determinations of the grown mycelia. Average yields and standard errors were calculated.

RESULTS

Growth of hyphal fragments in standard liquid F medium is restricted to the development of small, very thin mycelia. This is called regeneration. Development into strongly growing mycelium occurs when a root is present or when the incubation atmosphere is enriched with 0.5% CO_2 (Straatsma & Bruinsma, submitted; Straatsma et al., submitted).

In preliminary experiments with hyphal fragments of strain S1 many possible products resulting from carboxylating reactions were tested. Substances were tested separately or in combinations. In some experiments successful growth was obtained. However, growth patterns were characterized by long lag phases of about 60 days. Effects were ascribed in particular to malate, thymine and cytosine, but also to Tween 80, aspartate and glutamate.

Growth of hyphal fragments of strain S1 with a short lag phase occurred in F medium containing a combination of cytosine and thymine (0.1 mM), malic, glutamic, and aspartic acids (1 mM) and Tween 80 (10 µl/l). After 26 days this medium resulted in the production of 8.1 $\pm$ 0.8 (n=4) mg mycelium per flask. This mixture was tested for its constituents. Thymine and malic acid turned out to be essential. Cytosine, glutamate and aspartate had no effect (Table 1). Apparently Tween 80 had only a slight effect, therefore also higher concentrations had to be tested.

Table 1. Effect of the omission of constituents of the preliminary developed mixture (* cytosine and thymine (0.1 mM), malic, glutamic, and aspartic acids (1 mM), and Tween 80 (10 µl/l)). Yield determination after 32 days.

medium	Yield mg ± S.E.(n)
mixture*	6.8 ± 0.6 (2)
minus cytosine	8.0 ± 1.0 (4)
thymine	0.6 ± 0.1 (4)
Tween 80	4.0 ± 3.0 (3)
malic acid	1.1 ± 0.4 (5)
glutamate and aspartate	7.3 ± 0.7 (5)
control (F medium)	0.1 ± 0.1 (5)

In the next experiment malic acid, thymine, and Tween 80 were basically used at concentrations of 1 mM, 0.1 mM, and 10 µl/l, respectively. Tween 80 showed a strongly positive effect at higher concentrations (Table 2). In addition this experiment gives an impression of the variation in yields between different batches of a particular nutrient solution. Probably none of the three additions is strictly indispensable for growth, in the absence of one of them a long lag phase will occur. This is illustrated by the low yield (i.e. starting growth) in the absence of Tween 80. The inhibitory effect of 0.3 mM thymine was not further analyzed.

Table 2. The effect of different concentrations of malic acid, thymine, and Tween 80 on growth after 36 days of incubation. Reference concentrations were: 1 mM malic acid, 0.1 mM thymine, and 10 µl/l Tween 80 (media 3, 7, and 10).

medium	Yield mg ± SE (n)
0 mM malate	0.1 (5)
0.3	1.7 ± 0.6 (5)
1	4.5 ± 1.3 (4)
3	3.0 ± 1.6 (5)
0 mM thymine	0.2 (5)
0.03	1.4 ± 0.5 (5)
0.1	2.7 ± 1.1 (5)
0.3	0.1 (5)
0 $\mu l.l^{-1}$ Tween 80	1.5 ± 0.5 (5)
10	2.0 ± 1.3 (4)
30	14.3 ± 1.0 (5)
100	13.7 ± 0.9 (5)

In subsequent experiments with hyphal fragments of strain S1, F medium was supplemented with Tween 80 at 50 µl/l and with malic acid and thymine at 1 mM and 0.1 mM, respectively. This medium will be called Fc medium. Purines (guanine, adenine, and hypoxantine) were tested at concentrations of 0.1 and 0.01 mM in Fc medium. Hypoxantine had no effect; guanine and adenine were inhibitory. Additions to Fc medium of arginine, malonic, and mevalonic acids had no effect. Malic acid could be replaced by succinic and fumaric acids; oxaloacetic acid was inhibitory. Thymine could be replaced by uracil; orotic acid and arginine had no effect.

Hyphal fragments of strain T6 reacted to Fc medium in the same way as strain S1.

Emersed growth on Fc medium solidified with autoclaved agar to a final concentration of 1.2% (w/v) was successful. However, regeneration of hyphal fragments occurred only when they were initially covered with a silicone sheet. The effect of covering was already mentioned in experiments with CO_2 (Straatsma et al., submitted).

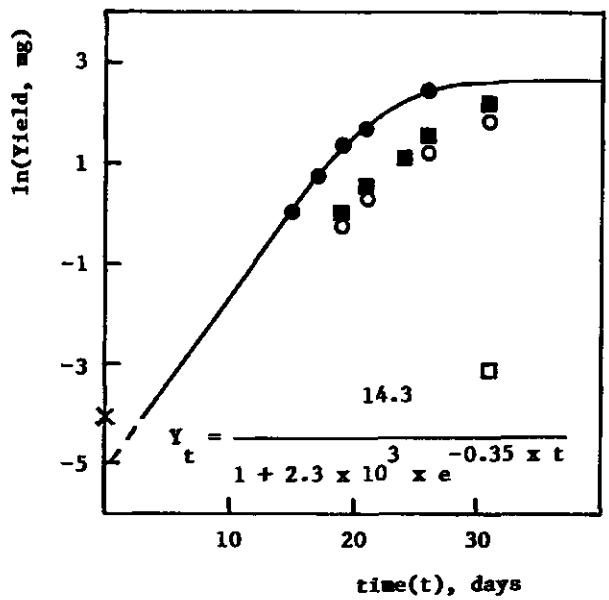
In a first control experiment, growth of mycelial tufts of strains S1 and T6 was studied in F and Fc medium. Growth in both media was identical. This reflects the independent growth of mycelial tufts of living roots or exogenously supplied CO_2 (Straatsma & Bruinsma, submitted; Straatsma et al., submitted).

In a second control experiment, hyphal fragments were tested in F and in Fc medium in the presence or absence of a living tomato root. No growth occurred in F medium in the absence of a root. Growth in F medium in the presence of a root and in Fc medium in the absence of a root was practically identical. Growth in Fc medium in the presence of a root was characterized by a (further) reduced lag phase (Figure 1). This result was probably caused by a positive effect of Fc medium on root fitness; this was indicated by a whiter colour of the roots in Fc medium. The data presented in Figure 1 show, as expected, no effects on the specific growth rate of C. cibarius.

DISCUSSION

As mentioned in the introduction the metabolic fate of fixed CO_2 can be quite divers. In nutritional studies it seems difficult to replace CO_2 by carboxylated products of the mentioned pathways (Gitterman & Knight, 1952;

Figure 1. Fitted logistic function of dry weight yields (Y_t) of C. cibarius strain S1 in Fc medium in the presence of a root (solid circles). In addition yields are plotted of growth in Fc medium in the absence of a root (hollow circles), of growth in F medium in the presence (solid squares) respectively in the absence (hollow square) of a root.



Buston et al.,1966; Boasson & Shaw,1984). In this study CO_2 could be replaced by a combination of malic acid, thymine, and Tween 80. These substances, or related products, are known to be synthesized after carboxylating reactions. This indicates that CO_2 plays a nutritional role during vegetative growth of C. cibarius. In correspondence with the nature of the effect of CO_2 on growth, the mentioned substances only reduce the length of an otherwise indefinite lag phase.

Effects of the mentioned metabolites or related substances on fungi were reviewed at length by Fries (1961, 1965). Amino acids and nucleic acid constituents can be "(growth) promoting" and "merely stimulatory", which indicates that they affect lag phases in particular. Growth stimulating effects of purines and pyrimidines were recently reported by Diner & Mott (1982) for the white-pine blister rust Cronartium ribicola.

Succinic acid successfully replaced CO_2 in growth experiments with Aqualinderella fermentans (Emerson & Held, 1969). However, the authors

ascribed an important pH buffering effect to succinic acid. The interferences of such indirect effects with direct effects of organic acids, especially fumaric acid, on nutrition are also discussed by Hacskaylo et al. (1954). The F medium used in our experiments contains 5.4 mM tartrate as a pH buffer. The addition of 1 mM malic acid can have only a slight effect on the buffering capacity. It is remarkable that glutamate and aspartate had no effects in our experiments. These amino acids are often labeled when fungi are exposed to $^{14}CO_2$ (Buston et al., 1966; Hartman et al., 1972; Rast et al., 1976; Boasson & Shaw, 1984). They stimulate growth of many mycorrhizal fungi (Melin, 1963). The successful replacement of glutamate and aspartate by a-ketoglutarate and fumarate in growth experiments with mycorrhizal Tricholoma species (Norkrans, 1953) indicates a reduced Krebs cycle activity rather then a growth-limiting nitrogen metabolism. In turn, this points to an anaplerotic function of these amino acids, which agrees with Norkrans remark that effects are "bound up with initial stages of growth".

Interesting is the report of Lindeberg & Lindeberg (1974) about a growth promoting effect at low concentrations of the fatty acids octanoate and decanoate on the mycorrhizal fungus Boletus (Suillus) variegatus. Growth stimulating effects of safflower oil on some Boletaceae were reported by Schisler & Volkoff (1977). Higher fatty acids also have a stimulatory effect on mycelial growth of the edible mushroom, Agaricus bisporus (Wardle & Schisler, 1969). A growth promoting effect of Tween 80 on A. bisporus is mentioned by Dütch (1978).

In our experiments, carbon dioxide could be replaced by a combination of malic acid, thymine, and Tween 80. These substances are associated with three metabolic pathways in which CO_2 fixation occurs: anaplerotic replenishment of oxaloacetic acid, pyrimidine synthesis, and fatty acid synthesis. The remaining pathways are arginine and purine syntheses. Arginine synthesis requires carbamoyl phosphate as precursor as does pyrimidine synthesis; arginine synthesis could be quantitatively less important. Perhaps purine synthesis depends on an enzyme which strongly competes for CO_2. However the importance of this pathway was hardly studied.

The three pathways limit strong growth of loose hyphae of C.cibarius and probably also of germinating spores.In nature, respiring roots may supply CO_2 to these pathways. In dense mycelia, respiration supplies sufficient CO_2 for their functioning.

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ALGEMEEN OVERZICHT EN DISCUSSIE

De belangrijkste resultaten van het in dit proefschrift beschreven onderzoek zijn het tot stand komen van een collectie reincultures van de cantharel en de beschrijving van de voedingsbehoeften van het mycelium. Een terugkoppeling tussen de resultaten van het onderzoek naar de omstandigheden van myceliumgroei en de werkwijze om reincultures te maken is nog niet tot stand gebracht. Zo is er nog geen onderzoek verricht om de sporekiemingsfrequentie te verhogen. Daardoor zijn ook de mogelijkheden voor (klassiek) genetisch werk, zoals het kruisen van monosporeculturen, nog niet bestudeerd.

Met behulp van koolzuur, van grote betekenis voor de groei van hyfefragmenten, kon homogeen groeiend mycelium verkregen worden. Het bleek tot dusverre niet mogelijk de relatieve groeisnelheid van mycelium te verhogen, anders dan door frequent overenten.

De vondst dat koolzuur een zo belangrijke rol speelt bij in vitro-kweek van cantharelmycelium, werpt een nieuw licht op het veronderstelde mycorrhizavormende karakter van deze paddestoel. In tegenstelling to de opvatting van Melin (1963), blijken wortels geen specifieke groeistimulerende functie te hebben. Onder natuurlijke omstandigheden zal door de ademhaling van wortels voldoende koolzuur aan het worteloppervlak aanwezig zijn. Tezamen met andere wortelexudaten en de in de bodem aanwezige mineralen, is daarmee in de voedingsbehoeften van cantharelmycelium voorzien.

Het in kweek brengen van het mycelium van de cantharel is een eerste stap in de domesticatie van deze eetbare paddestoel. De cultures vormen de basis voor verder morfogenetisch onderzoek. De voor succesvolle domesticatie benodigde studie naar mechanismen voor knopvorming is een tweede stap in het programma. Daarvoor is in dit proefschrift de basis gelegd. Tijdens het onderzoek is geen spontane vorming van vruchtlichamen in de kweken opgetreden. Er laat zich dan ook geen uitspraak doen over omstandigheden die daartoe zouden kunnen leiden.

Isolatie en reincultuur

Het maken van reincultures, beschreven in Hoofdstuk 1, was technisch gezien niet moeilijk. Reincultures werden uit twee soorten uitgangsmateriaal verkregen, namelijk uit sporen en uit paddestoelweefsel. Er is relatief veel materiaal nodig om cultures te maken. Dit wordt veroorzaakt door het geringe aantal sporen dat bij de aangelegde omstandigheden kiemt en door het lage

aantal stukjes vruchtlichaamweefsel dat niet overgroeid wordt door bacteriën. De incubatietijden van ongeveer drie maanden zijn erg lang. Tomatewortels bleken de meest geschikte stimulator voor sporekieming te zijn. Isolatie uit stukjes paddestoelweefsel lukte vooral op een gemodificeerd Murashige & Skoog (M&S) medium.

Ons criterium voor sporekieming was niet scherp gedefiniëerd, omdat alleen gelet werd op het ontstaan van groeiende kolonies. Er kunnen twee fasen in het proces van het ontstaan van kolonies uit sporen worden onderscheiden. Het doorbreken van de kiemrust (*dormancy*) (Griffin, 1981) is de eerste fase; de uitgroei is de tweede fase. Het bestaan van kiemrust in sporen van mycorrhizaschimmels is aannemelijk omdat veel soorten een specifieke stimulatie door soorteigen mycelium of door een gastheerwortel nodig hebben (Fries, 1983). De chemische(?) achtergrond van deze kiemrust is echter nog onbekend. Bjurman & Fries (1984) hebben het GIF (germination inducing factor), werkzaam bij Leccinum aurantiacum, de rosse populierboleet, geïsoleerd en gezuiverd. De verdere analyse van GIF en het ophelderen van zijn metabolische rol kan belangrijk zijn.

Aangezien tomatewortels een sterk stimulerend effect hebben op de groei van fijn gefragmenteerd mycelium (zie Hoofdstuk 3), zullen ze zeker een rol spelen tijdens de tweede fase van de kieming. Hiermee samenhangend kan de zeer lage kiemingsfrequentie verklaard worden door te veronderstellen dat de kiemrust in een aantal gevallen spontaan doorbroken is. Onderzoek naar de voorwaarden voor groei van hyfefragmenten (zie Hoofdstuk 3 tot en met 5) heeft een grote betekenis voor het onderzoek naar sporekieming.

Isolatie uit stukjes paddestoelweefsel lukte alleen als de stukjes niet overgroeid werden door bacteriën. De aanwezigheid van een wortel in het isolatiemedium was geen voorwaarde voor isolatie. Hoewel het M&S-medium niet optimaal is voor de groei van cantharelmycelium (Hoofdstuk 2), was het geschikt om de groei van bacteriën - aanwezig in paddestoelweefsel - te verhinderen. De selectiviteit van het medium, die waarschijnlijk gebaseerd is op de koolhydraatbron, was veel groter dan de selectiviteit van Fries-medium met antibiotica.

De afwezigheid van een worteleffect op het isolatiesucces is niet verwonderlijk. In Hoofdstuk 3 blijkt dat wortels wel een effect hebben op de groei van hyfefragmenten, maar niet op die van stukjes mycelium. De overeenkomst tussen een stukje paddestoelweefsel en een stukje mycelium is duidelijk.

De DNA/DNA hybridisatie-experimenten leverden een uitermate betrouwbaar

criterium voor de (juiste) identiteit van de getoetste stammen. DNA/DNA hybridisaties discrimineren zeer sterk tussen verschillende soorten, zoals ook blijkt uit ander onderzoek met paddestoelen (Horgen et al., 1984). De mogelijkheid om verwantschappen vast te stellen tussen soorten binnen één geslacht of hoger taxon, lijkt beperkt. Wellicht zijn daarvoor DNA/rRNA hybridisaties meer geschikt, zoals dat bij een aantal gisten beschreven werd (Kurtzman et al., 1983).

Er bestaan verschillen tussen de geïsoleerde stammen. Gegevens over deze variatie, met betrekking tot groei, zijn te vinden in Hoofdstuk 3.

De slechte groei van een aantal stammen zou verklaard kunnen worden door het ontbreken van bepaalde groeifactoren in de gebruikte voedingsoplossing. De mogelijkheid bestaat echter dat deze stammen gevoeliger zijn dan de stammen die uitgebreid getoetst zijn. Deze gevoeligheid komt in Hoofdstuk 2 aan de orde.

Het is van groot belang dat de geïsoleerde stammen hun genetische eigenschappen behouden, met andere woorden niet degenereren. Mycorrhizavormende schimmels die in reïncultuur gehouden worden, zouden na verloop van tijd hun vermogen om planten te infecteren kunnen verliezen (Molina & Palmer, 1982). De stammen S1 en T6 bleken beide in de loop van de tijd een hogere relatieve groeisnelheid te vertonen, die onafhankelijk van de experimentele omstandigheden was (Hoofdstuk 2). Daarnaast verloren hyfefragmenten van stam S1 geleidelijk hun vermogen om te regenereren bij emerse incubatie.

Voedingsbehoeften van mycelium

In Hoofdstuk 2 wordt verslag gedaan van experimenten over de voedings- en incubatiebehoeften van twee stammen. Het is nuttig te onderstrepen dat deze experimenten gedaan werden met stukjes mycelium; dit in tegenstelling tot het latere werk met fijn gefragmenteerde hyfen.

Het vanaf het begin gebruikte gemodificeerde Fries medium en de gebruikte incubatie omstandigheden bleken vrijwel optimaal. Sterilisatie van het voedingsmedium moet door filtratie gebeuren; dit is een standaard techniek geweest in het hele onderzoek. De buffering van het medium zou iets beter kunnen door het vervangen van tartraat door succinaat of MES (morfolinoethaan-sulfonzuur). Deze vervanging is niet toegepast in het verdere onderzoek, omdat dit al ver gevorderd was.

De groeibehoeften van de cantharel zijn analoog aan die van andere mycorrhizavormende schimmels. De groeisnelheid is echter laag en de gevoeligheid

van het mycelium is groot. Dit laatste blijkt onder andere uit de groeiremming veroorzaakt door filtreerpapier en watteproppen.

Het bleek dat de relatieve groeisnelheid, waaraan de verdubbelingstijd is gekoppeld, niet te verhogen viel door voeding of incubatie. Regelmatig overenten doet blijkbaar de groeisnelheid wel toenemen.

Extra-chromosomale veranderingen kunnen een rol spelen bij het onstaan van variaties in de groeisnelheid (Jinks, 1957). Waarschijnlijk komt het verschijnsel van een hogere groeisnelheid naar voren door een onbewust toegepaste selectie op de uitgesneden stukjes mycelium die voor het overenten worden gebruikt.

Lage opbrengsten waren niet te wijten aan een lage groeisnelheid maar aan een verlaagde maximum opbrengst. De oorzaak hiervoor was een vervroegde ophoping van afbraakprodukten in het medium.

Er was verschil in groei tussen mycelia die óp (emers) respectievelijk ónder (submers) het medium oppervlak werden geïncubeerd. Emerse incubatie resulteerde in hogere opbrengsten; de vertakkingsgraad van emers groeiend mycelium was hoger. Submerse incubatie was echter meer geschikt om de groei te laten beginnen. Bij submerse groei zou er sprake kunnen zijn van een geremde gasuitwisseling. Hierdoor kan zich koolzuur ophopen wat gunstig is bij de start van de groei (Hoofdstuk 4). Waarom het afdekken van hyfefragmenten, een andere vorm van submerse incubatie, ook bij 0.5 % koolzuur gunstig werkt (Hoofstukken 4 en 5), is echter onduidelijk. Wellicht spelen ook andere mechanismen een rol.

Voedingsbehoeften van hyfefragmenten

In Hoofdstuk 3 wordt de invloed van levende wortels op groei van hyfefragmenten beschreven. Zonder wortels regenereren hyfen tot kleine, ijle mycelia die, in het geval van stam S1, daarna ophouden te groeien. Als er een wortel in het medium aanwezig is, gaat de groei door en ontstaan normale, dichte mycelia. Niet alle 14 getoetste stammen reageren op dezelfde manier; twee stammen groeien zonder wortels net zo goed als met wortels. De wortels hebben geen effect op de relatieve groeisnelheid. Drie gegevens wijzen er op dat de door tomatewortels geproduceerde factor ook door de schimmel zelf gemaakt wordt:

- stukjes mycelium in de afwezigheid van een wortel groeien met dezelfde relatieve groeisnelheid als hyfefragmenten in aanwezigheid van een wortel;
- bij groeicurves van hyfefragmenten van een aantal stammen is te zien dat de

aanwezigheid van een wortel alleen effect heeft op het moment waarop de groei begint;
- hyfen die in nauw onderling contact werden gebracht groeiden daardoor net zo als een stukje mycelium.

Blijkbaar is er sprake van een basale groeisnelheid, die niet door wortels beïnvloedt wordt. De lage groeisnelheid van de cantharel is hiermede een vaststaand gegeven. De lengte van de verdubbelingstijd, ongeveer twee dagen, kan model staan voor de lengte van de celcyclus. Het is niet duidelijk hoe de duur van deze cyclus gereguleerd is. Een en ander heeft onder meer te maken met de snelheid waarmee de DNA- synthese na celdeling tot stand komt (Wessels, 1983).

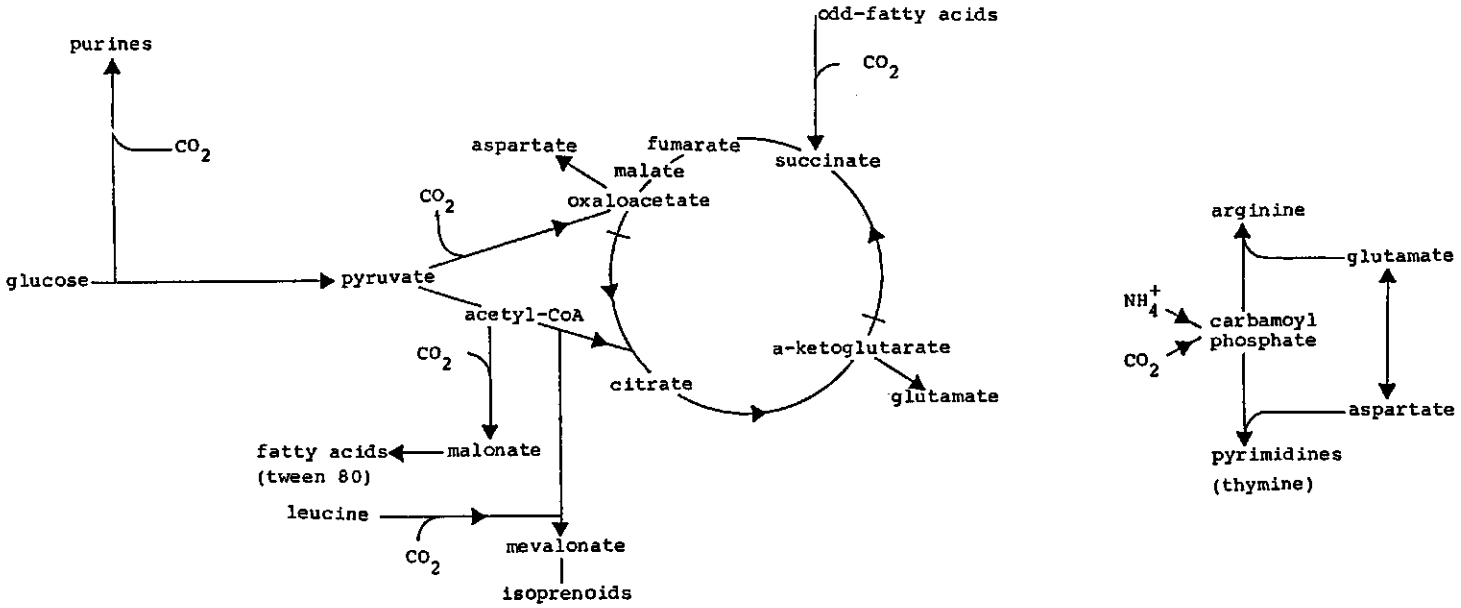
In Hoofdstuk 4 worden proeven beschreven die duidelijk maken dat de nog onbekende groeifactor voor hyfefragmenten vluchtig is. De stof wordt niet alleen door wortels en soorteigen mycelium geproduceerd, maar ook door andere schimmels. Het blijkt om koolzuur te gaan. Aanvulling van de incubatie-atmosfeer met koolzuur tot 0,5% is voldoende.

Koolzuur is een gewone groeifactor. Deze wordt meestal over het hoofd gezien omdat groeiend en ademend mycelium normaal gesproken ruimschoots in eigen behoefte voorziet (Griffin, 1981). In samenhang met Hoofdstuk 2 blijkt hoe simpel de voedingsbehoeften van het cantharelmycelium in principe zijn. De verwachting dat deze schimmel - omdat het een mycorrhizavormer is - zeer specifieke voedingseisen heeft, blijkt niet gegrond.

Het uitgevoerde onderzoek geeft mede inzicht in de voorziening met en het succesvol gebruik van deze groeifactoren onder natuurlijke omstandigheden. De produktie van koolzuur door de wortel kan een belangrijke factor zijn bij de vestiging van het mycelium. Het is nog niet duidelijk hoe de cantharel in zijn behoeften kan voorzien in concurrentie met andere bodemschimmels die minder gevoelig zijn en sneller groeien.

In Hoofdstuk 5 wordt aannemelijk gemaakt dat de inbouw van koolzuur in drie verschillende stofwisselingsroutes limiterend is voor groei van hyfefragmenten. Het toevoegen van produkten uit deze routes maken een goede groei zonder koolzuur mogelijk. Het gaat om een zogenaamde anaplerotische route die de Krebs-cyclus van (uiteindelijk) oxaalazijnzuur voorziet, om de pyrimidine- en de vetzuursynthese (zie Figuur 1). Metabolieten die samenhangen met deze routes zijn bij veel schimmels werkzaam als groeifactor. Een onderling

Figuur 1. Heterotrofe CO_2 fixaties in het metabolisme.



verband tussen deze groeifactoren en hun betekenis voor het metabolisme van die schimmels blijft meestal onduidelijk.

Het onderzoek beschreven in de Hoofdstukken 2 tot en met 5 laat een duidelijk samenhangend beeld zien. In het kort: stukjes mycelium groeien onafhankelijk van de factor(en) die nodig zijn om de groei van hyfefragmenten mogelijk te maken. Deze factor(en) heeft (hebben) geen invloed op de relatieve groeisnelheid, omdat ze door groeiend mycelium zelf geproduceerd wordt (worden). De sleutelfactor in het geheel is koolzuur dat in een aantal centrale stofwisselingsroutes nodig is.

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SUMMARY

In this thesis in vitro growth of mycelium of Cantharellus cibarius Fr. is described as the first step in the domestication of this edible mycorrhizal mushroom. A collection of pure cultures was obtained and the requirements for vegetative growth were determined.

Isolation

A culture collection of 33 strains was obtained by isolation from spores or by cultivation from fruit body tissue. Isolation was technically not difficult. However, many attempts were required because only very few spores germinated and only few tissue explants remained free from contaminants. In addition the incubation time required for successful isolation is long, viz. three months. DNA/DNA hybridization experiments proved the identity of the cultures. The biological variation in the strain collection was illustrated by differences in growth parameters such as relative growth rate.

Growth requirements

The objects of the experiments were the development of an optimal nutrient solution for mycelial growth and the elucidation of root effects on growth. In these studies two kinds of inoculum were used: mycelial tufts and hyphal fragments. These inocula showed different requirements. Mycelial tufts grew strongly in Fries medium supplemented with micro- minerals. Hyphal fragments showed more extensive requirements; their growth was limited to the development of small and thin mycelia.

The modified Fries medium and incubation at 20°C appeared almost optimal for growth of mycelial tufts. In sterilization of the nutrient solution, filtration is of major importance. Emersed growth was found to be better than standard, submersed incubation. Fungal growth reacts strongly to various additions to the medium. The specific growth rate was low. It could not be increased by the use of other media or by addition of possible growth factors to modified Fries medium. The specific growth rates of the tested strains increased in the course of the studies independently of nutrition or incubation conditions.

Development of hyphal fragments into strongly growing mycelium at first required the presence of a living root in the nutrient solution. Three observations indicated that the factor(s) involved could be produced by the

fungus itself:
- the specific growth rates of mycelial tufts in the absence of roots and of hyphal fragments in the presence of roots were identical;
- kinetics of growth of hyphal fragments showed that a root effect was limited to the duration of the lag phase;
- hyphae brought into intimate mutual contact grew as strongly as a mycelial tuft.

The unknown growth factor(s) was volatile in nature. It could be replaced by carbon dioxide. A 0.5% concentration of CO_2 in the incubation atmosphere is sufficient for strong growth of hyphal fragments.

CO_2 could be replaced by a mixture of malic acid, thymine, and Tween 80. This suggests that CO_2 fixation reactions involved in the replenishment of Krebs cycle intermediates, in the synthesis of pyrimidines, and in the synthesis of fatty acids are growth limiting for hyphal fragments.

On the one hand, the homogeneous growth of hyphal fragments, described in this thesis, allows the second step in the domestication of this mushroom, viz. the study of fruit body formation. On the other hand, the analysis of the growth requirements of these fragments further elucidates the ecological nature of the mycorrhizal relationship between C. cibarius and plant roots. The view that the fungus is supplemented with specific growth factor(s) is not supported. The production of carbon dioxide by roots enables the establishment of the fungus in the rhizosphere.

LEVENSOVERZICHT

Gerben Straatsma werd op 14 augustus 1955 in de gemeente Barneveld geboren. In 1972 behaalde hij het diploma HBS-B op het Ichthus College in Drachten. Daarop aansluitend begon hij aan een biologiestudie aan de Landbouwhogeschool. In september 1979 behaalde hij zijn doctoraal diploma, met als eerste hoofdvak vegetatiekunde, als tweede hoofdvak natuurbeheer en als bijvak fytopathologie.

In januari 1980 kwam hij in dienst van het Ministerie van Landbouw en Visserij en begon hij, op het Proefstation voor de Champignoncultuur te Horst, aan het onderzoek over de cantharel. In eerste instantie had hij een stageplaats en vanaf december 1982 is hij in vaste dienst.