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Formation of sclerotia by Rhizoctonia solani on artificial media and potato tubers

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Abstract

Independent of the nutrient medium and the size of Petri dish, sclerotium initials of *Rhizoctonia solani* AG-3 appeared after a fixed period of time. The same held for the completion of maturation of the sclerotia. Deprivation or extra supply of nutrients reduced or increased, respectively, final mass of sclerotia but did not affect the moment of initiation and of maturation. Transfer of a mycelial mat from water agar to a nutrient-rich medium caused the formation of black solid sclerotia within four days all over the mat and not only around the site of inoculation as usually occurs on rich medium. Final sclerotial mass was higher on liquid medium than on agar. The results indicate that formation of these sclerotia is not associated with cessation of linear hyphal growth or with starvation.

On mature tubers, sclerotia and hyphae are spread over the whole surface. On young growing tubers sclerotia are rarely found in the immediate vicinity of lenticels. This suggests a release of inhibitory factors at these sites which diminishes during tuber maturation. Volatile exudates from underground plant parts seem to further promote the sclerotium formation. On all underground plant parts, even the roots, final sclerotial mass was higher after wounding.

After haulm destruction, development of black scurf was not stimulated by a short-term trigger or by roughning of tuber surface. The observations rather suggest that the stimulation results from an increased tuber exudation of stimulatory nutrients, water and stress factors and also from a reduction of as yet unknown inhibitory factors. Results indicated that in infested soils, the estimated inoculum density at the day of haulm destruction has no predictive value for black scurf development.

Additional keywords: black scurf, haulm destruction, sclerotial distribution, sclerotial initiation, sclerotial maturation, *Solanum tuberosum*, tuber maturation, tuber surface.

Introduction

Quantitative and qualitative alterations in nitritive components and pH affect production of pseudo-sclerotia by *Rhizoctonia solani* Kühn AG-3 both in their number, size and location on artificial media (Allington, 1936). the effect of nutrients on mycelial growth and sclerotial initiation differs from their effect on sclerotial growth and maturation (Townsend, 1957). Volatile compounds also affect the final mass of sclerotia pro-

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duced (Sherwood, 1970; Lewis, 1976). Spontaneous sclerotium production and effective minimum concentrations of adenosine 3',5'-cyclic monophosphate (cAMP) to induce sclerotium formation, depend on the age of mycelium (Hashiba and Ishikawa, 1978). The authors also suggested that environmental factors play a role.

Potato tubers stimulate black scurf when tuber maturation is accelerated after haulm destruction (Dijst, 1985). This effect may be caused by an increase in stimulatory factors, a reduction of inhibitory factors or by a short trigger. Armentrout et al. (1986) suggested that surface structure influences sclerotium production. Allington (1936) concluded that tubers, apart from providing surface area, offer nutrients. The objective of this study was to obtain more information about factors that stimulate black scurf formation after haulm destruction. For that purpose, sclerotial development was studied on artificial media and on tubers. An attempt was made to determine the moment of stimulus release by the tuber after haulm destruction.

Materials and methods

Isolates. Three pathogenic isolates of *R. solani* Kühn AG-3 of different origin were provided by G. Jager (Inst. of Soil Fertility, Haren, the Netherlands): 09ABa from loamy clay, pH 7; 05AHa from sand, pH 4.5; 36AG78 isolate from a symptomless plant on loamy clay, pH 7.0. Over five years of experiments these isolates gave similar results in tests on sclerotium formation, although their distribution pattern on plates differed.

Tests in vitro. Isolates were maintained on malt peptone agar (MPA) (Van den Boogert and Jager, 1984) and tested on water agar (WA). Cultures were incubated at 20 °C. Disks, 5 mm in diameter, were cut from the edge of two-day-old MPA cultures and placed on WA. After two days, small disks with hyphal tips from these WA-cultures were used to inoculate filters or plates.

Growing of plants. Cultivar Pimpernel was used for its long stolons which facilitate working with growing tubers in pot experiments. Shoot tops were cut from one-month-old plants grown in growth chambers. Growth chamber conditions were kept at 16 h light per day and 18-20 °C by day and 14-16 °C during the night. Two weeks later, axillary stem cuttings (50 mm) were taken and placed in humidified perlite for two weeks. Rooted stem cuttings were transplanted into potting soil. After three weeks, the soil was washed from the roots in order to facilitate transplanting into a soil-sand system, a slight alteration of the previously described two-compartment system (Dijst, 1985). The lower compartment was a pot (180 mm) filled with potting soil and the upper compartment was a plastic bag of the same size. The roots of the transplants were placed onto the soil through a small hole in the plastic bag and the bags were filled with coarse sand. The original cut surface of the stems was in the sand, 20 mm above the plastic bottom in order to guarantee that all stolons and tubers were formed in the sand. Potting soil, sand and perlite were steamed twice before use. Pots were placed in the glasshouse on cotton mats, which were watered dayly. Extra light was supplied for 12 h a day during the winter and temperature was 18-20 °C by day and 14-16 °C at night. Experiments were started when plants were about 100 days old, shoots were still green and tubers were 30 mm. At the day of haulm destruction, plant shoots were either left untreated (UNTR) or cut off (COS).

Plant inoculation. Per pot, either the sand or each separate tuber was inoculated. Sand was inoculated with three sclerotized wheat grains per pot, placed at 4 cm depth when plants were two months old. The wheat grains had been soaked in water for 24 h, autoclaved twice with an interval of 36 h and inoculated. However, in order to assure equal infestation of all tubers per plant, eacht tuber was inoculated separately, two weeks before haulm destruction. For that purpose the sand was removed, each tuber was placed on two-day-old WA-cultures and covered with wet perlite until assessment of sclerotium production.

Assessment of sclerotium production. Three weeks after inoculation, sclerotium production was evaluated. Black scurf index was assessed as described previously (Dijst, 1985). Sclerotia were removed from tubers by hand and from artificial media by filtration after boiling the agar in acidified water. Sclerotium production was calculated per plate or per tuber as dry weight (μg) per 1000 mm^2 of surface area (WSA).

Estimation of tuber surface. Tuber length (l), tuber diameters at the middle (m_1 and m_2), at 10 mm from the bottom (b_1 and b_2) and at 10 mm from the top (t_1 and t_2) were measured and the average diameters at each site were calculated (m, b and t, respectively). If tuber length was shorter than 30 mm, tuber surface (A) was calculated as that of a sphere: $A = 4\pi r^2$ with $r = (b + m + t)/6$. If the tuber was longer, its surface was estimated as that of a cylinder in the middle (M) with half a sphere at each end (B and T):

$A = A_B + A_M + A_T$ with:

$A_B = 2\pi r_B^2$ with $r_B = (b_1 + b_2 + 20)/6$

$A_M = 2\pi r_M^2 (l - 20)$ with $r_M = (b + m + t)/6$

$A_T = 2\pi r_T^2$ with $r_T = (t_1 + t_2 + 20)/6$

Statistical analysis. Analyses of variance were carried out using GENSTAT-4 (Dijst et al., 1986). Differences between amounts of sclerotia produced (WSA) were analyzed after transformation to natural logarithm: ln(WSA + 1).

Results

Sclerotium production in vitro. The objective of this investigation was to repeat the study of Allington (1936) who suggested that sclerotium production starts at termination of hyphal linear growth. For this purpose cultures were compared on nutrient-richt agar (MPA), nutrient-poor agar (WA) and on liquid malt peptone (MP) in Petri dishes, 50, 90 and 150 mm in diameter. Isolates were similar in radial growth rate, viz. 10 mm per day on WA and 11 mm on MPA, which decreased slightly after five days. On WA a few hyphae developed with some branches (ca. seven hyphae per 10 mm, measured 10 mm from the site of inoculation). On MPA, dense colonies developed with concentric zones of heavily branched mycelium. All three isolates formed a few tiny sclerotia of about 1 mm in diameter on WA scattered over the plates. Isolates on MPA differed in distribution pattern of sclerotia. Isolate 09ABa produced many sclerotia as an almost solid mass extending not farther than 20 mm from the site of inoculation. Isolate 05AHa formed many sclerotia in a wide circle at 16 to 50 mm from the centre. Isolate 36AG78 produced many small sclerotia scattered over the plates, but never

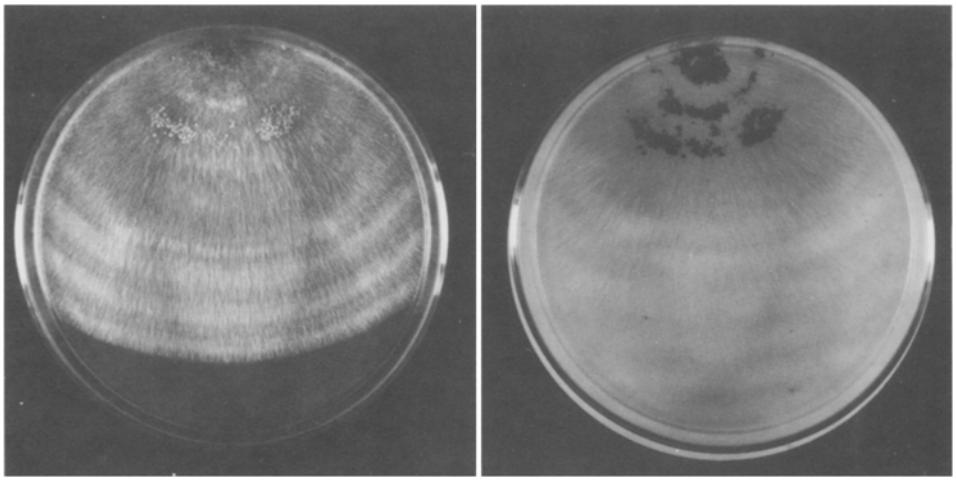

Fig. 1. Sclerotium formation by *R. solani* AG-3 on a 90-mm malt peptone agar plate at 20 °C: the same culture with immature light brown sclerotia at 6 days after inoculation (left) and with black mature sclerotia at 20 days after inoculation (right).

farther than 80 mm from the inoculation site. After 4 days of growth on agar media, white sclerotial initials were visible and at day 8 all sclerotia were black and solid regardless of dish size, type of medium or isolate tested (Fig. 1). Sclerotium production on MPA stopped at day 9, even when the colony had not yet reached the edge of the plate in the 150-mm dishes. Thus, on agar media, initiation and maturation of sclerotia was not associated with linear growth.

In addition to providing a surface, tubers probably offer nutrients that facilitate sclerotium production (Allington, 1936). Since black scurf stimulation is not visible until 7 to 10 days after haulm destruction, the time it takes before a response of *R. solani* can be observed on agar media after a sudden supply of nutrients was determined. On day 0, 90-mm Petri dishes with WA or MPA, were covered with a hydrophylic membrane filter (Sartorius SM 11107, cellulose acetate, 0.2 μm pore diameter) and inoculated with isolate 05AHa. On day 4 the filter with mycelium was transferred to either WA or MPA. Growth and sclerotium production were evaluated on day 8. Mycelium transferred from WA to WA or from MPA to MPA developed and produced sclerotia as described above. An increase in nutrient supply (transfer from WA to MPA induced formation of many hyphal branches and many sclerotia within the next four days. The mycelium formed a dense mat and sclerotia were scattered all over the colony and not restricted to the site of inoculation as is usally the case on MPA (Fig. 2: dish 5 and 6). Therefore, it is improbable that a local check of mycelial growth induces sclerotium formation on agar plates, as suggested by Townsend (1957). A reduction in nutrient supply (transfer from MPA to WA) reduced final sclerotium dry weight, but did not affect their location within the colony (Fig. 2: dish 7 and 10). Neither the time of initiation nor the period of time needed for maturation of the sclerotia was affected by these alterations in nutrient supply. Thus, on agar media, stimulation of sclerotium production occurred within 4 days after an increase in nutrient supply.

On liquid MP, a large black sclerotial crust, surrounded by smaller crusts, was form-

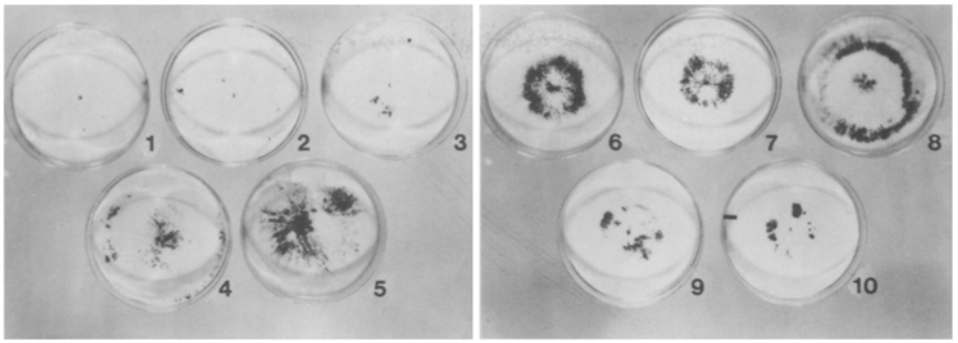

Fig. 2. Sclerotium formation by *R. solani* AG-3 isolate 05AHa (Rs) at 12 days after inoculation on day 0 on acetate cellulose membrane filters placed on either water agar (WA) or malt peptone agar (MPA) and transferred on day 4 to another plate. History of the plates:
1) inoculated on WA and kept on the same plate;
2) inoculated on WA and transferred to an empty dish;
3) inoculated on WA and transferred to a fresh WA plate;
4) inoculated on WA and transferred to an 'old'* MPA plate;
5) inoculated on WA and transferred to a fresh MPA plate;
6) inoculated on MPA and kept on the same plate;
7) inoculated on MPA and transferred to an empty dish;
8) inoculated on MPA and transferred to a fresh MPA plate;
9) inoculated on MPA and transferred to an 'old'* WA plate;
10) inoculated on MPA and transferred to a fresh WA plate.
* 'old' = previously used for 4 days by another Rs culture.

ed at the site of inoculation. These sclerotial crusts exuded large brown droplets and continued to grow for at least two weeks longer than those on agar media. Thus, availability of water also increased final sclerotium dry weight, altered sclerotial distribution pattern within the colony and did not affect the moment of sclerotial initiation nor the period of time needed for maturation.

In order to investigate possible effects by degeneration products, filter plus mycelium were removed from the agar plates after 10 days and the plates were re-inoculated. In general, the appearance of the second culture on the same plate, did not differ from the first one (see also Fig. 2: dish 4 and 5 as compared to 9 and 10). Continuous growth on the same plate resulted in sclerotia on and around the first dense zone, whereas transfer to fresh medium on day 4 produced sclerotia in the second dense zone (Fig. 2: dish 6 and 8). Therefore, sclerotium production does not seem to be induced by a local check of mycelial growth as suggested by Townsend (1957) nor by degeneration products.

Sclerotium formation on tubers. When growing tubers were inoculated with mycelium on WA, white initials became visible within 4 days and these became mature sclerotia within the next 4 days, similar to the pattern obseved on agar plates. In infested sand, black sclerotia gradually developed on the growing tubers and white initials were seldomly observed suggesting that maturation occurs more quickly in vivo than in vitro.

Cutting-off shoots (COS) stimulated not only sclerotium formation (Dijst, 1985), but also growth of mycelium on the tubers. At the time of haulm destruction, some

hyaline hyphae and a few tiny black sclerotia were found on infested tubers, which became embedded in a mass of mycelium in which the first brown hyphae appeared after 8 to 10 days. With each following day, fewer hyaline hyphae and more brown hyphae and black sclerotia appeared. The sudden production of hyphae and sclerotia after COS seemed analogous to the sudden production of sclerotia on agar all over existing mycelium after a supply of nutrients.

On young growing tubers, sclerotia were never observed on or beside lenticels. However, on older mature tubers and after COS, sclerotia were found all over the tubers, showing no definite preference or aversion for lenticels; and lenticels seemed to be 'explored' by runner hyphae (Fig. 3). This indicates that inhibitory factors may be reduced when tubers mature.

Furthermore, the study examined whether the stimulation was initiated by a single signal from the tubers shortly after COS. In a glasshouse experiment, tubers were inoculated at 0, 9 or 13 days after COS and were either left attached to the plant or their stolon was broken. The inoculated tubers were covered by wet coarse perlite with a plastic sheet folded loosely on top to keep the perlite moist. Data of two experiments were similar and are shown in Table 1. Sclerotium production on both attached and detached tubers was very high when incubated in pots with COS plants, low in pots with untreated plants, but very low in boxes without plant material. At harvest, water content

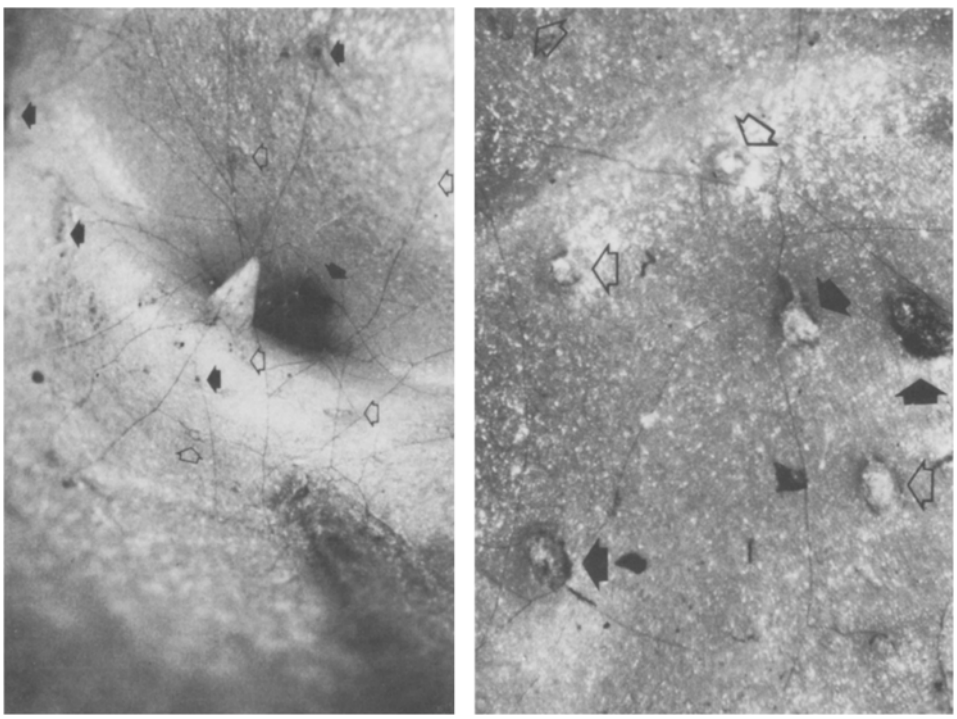

Fig. 3. Surface of mature tubers with lenticels (arrow) fully or partially sclerotized (black arrow) by *R. solani* AG-3 and brown runner hyphae around an eye (left) and, at higher magnification, elswhere at tuber surface (right).

Table 1. Final sclerotium dry weight of *R. solani* on potato tubers (ng mm^{-2}) after three weeks of incubation in boxes or in pots with plants with shoots either untreated or cut off; tubers remained attached to the plants or stolons were broken.

Plant treatment	Inoculation time[1]							
	tubers incubated (still attached or loose) in pots with plants						in a box without plants	
	one tuber/pot				two tubers/pot			
	0	9	13	13	13		9	13
	att.	att.	att.	loose	att.	loose[2]	loose	loose
Experiment 1[4]								
Shoots untreated	473 b[3,4]	154 b	—	—	—	—	112 b	—
Shoots cut off	6969 a	10329 a	—	—	—	—	166 b	—
Experiment 2[5]								
Shoots untreated	—	—	701 cd[3,5]	710 bc	661 cd	1183 bc	—	282 d
Shoots cut off	—	—	3052 a	2271 a	1435 ab	1851 ab	—	76 e

[1] Number of days after the day of haulm destruction.
[2] Loose tuber taken from plants of same age where shoots had been cut off.
[3] Analysis of variance was carried out per experiment over ln(WSA + 1) and indicated differences were either not significant (NS) or significant at $P = 0.05$ (*), $P = 0.01$ (**) or $P < 0.01$ (***).
[4] Per 'Tuber treatment' (incubation in plant pot from day 0 or 9, or in box from day 9) values followed by the same characters are not significantly different at $P = 0.05$, significances being **, *** and NS, when testing 14, 10 and 25 tubers, respectively.
[5] Split-plot design, comparing 15 tubers per plant treatment × tuber treatment combination. Significances: plant treatment = NS; tuber treatment = ***; plant treatment × tuber treatment = ***. Values followed by the same characters are not significantly different at $P = 0.05$.

of the perlite was the same in the plant pots and in the separate boxes suggesting that volatile exudates of underground plant parts affect black scurf development. On a loose COS tuber incubated near an attached UNTR tuber less sclerotia developed (1183) then when incubated in a separate pot (2271). Black scurf development on the UNTR tuber was not affected by the near presence of a loose COS tuber. Thus, growing tubers seem to exude a volatile inhibitor which can suppress the stimulation of black scurf on COS tubers. All results were identical regardless of the time of inoculation. Thus, the possibility that a short term trigger is involved seems unlikely.

Influence of damage and wounding on black scurf formation. When grown in infested perlite, enormous amounts of sclerotia were produced on all underground potato plant parts, including roots. Tuber periderm appeared to be damaged by the sharp granules. In order to investigate the effect of alterations in surface structure on black scurf development, tubers were partly wounded by stripping off the periderm while they were still attached to the plant. Wound periderm is produced within a few days where periderm is removed (Artschwager, 1927). Sclerotia remained immature on wounded sites regardless of plant treatment. On UNTR tubers, dry weight of sclerotia was less on wounded than on unwounded sites. On COS tubers, wounded sites often became completely

covered by a solid layer of immature sclerotia. This shows that growth and maturation of sclerotia are affected differently. For mature sclerotia only, black scurf index on unwounded and on partly wounded tubers was 4 and 13, respectively, with UNTR tubers and 6 and 33, respectively, with COS tubers. Thus, on damaged tubers the production of mature sclerotia is enhanced.

Discussion

Sclerotium production in vitro. Allington's observation (1936) that sclerotium production starts after termination of linear hyphal growth does not apply to the isolates used in my experiments. No effect of degeneration products was observed on agar media, which confirmed the results of Allen and Haenseler (1935). Neither restriction of linear growth nor local inhibition of hyphal growth nor deprivation of nutrients stimulated sclerotium formation by *R. solani* AG-3. Thus, sclerotium production is not associated with starvation as has been reported for other sclerotium-forming fungi (Townsend, 1957; Chet and Henis, 1975).

By others (Wheeler and Walker, 1965; Christias and Lockwood, 1973) deprivation of nutrients has been reported to induce sclerotium formation on liquid media both in *R. solani* and in other sclerotium-forming fungi, while a supply of nutrients delayed it. In my experiments, however, a decrease of nutrients reduced the amount of sclerotia and a supply enhanced it; the time of initiation and the speed of maturation were not affected.

It is not yet possible to explain the discrepancies in the results observed by the various research workers. Christias and Lockwood (1973) used a different medium, namely potato dextrose broth, which may be less suitable for studying the development of sclerotia by *R. solani*. In my experiments, isolates kept on MPA still produced mature sclerotia after five years, but not if kept on PDA. Furthermore, my isolates behaved differently on liquid than on agar media. Townsend (1957) reported that it is not the total amount, but the composition of nutrients which plays a role.

Sclerotium formation on tubers. Tuber physiology and environmental conditions influence development of black scurf (Dijst, 1985). During tuber maturation, several factors influencing black scurf development may change. The production of sclerotia on wound periderm and on artificial medium indicates that the surface structure is not a major critical factor, as suggested by Armentrout et al. (1986), although skin roughening may facilitate it. On young growing tubers, inhibiting exudates may reduce the formation of sclerotia around lenticels. Stimulatory circumstances may be created by mature tubers. This can be inferred from the fact that black scurf was not significantly reduced on loose mature tubers that were placed close to growing tubers, which seem to produce inhibitors. Finally, volatiles from underground plant parts may facilitate sclerotium formation, since more black scurf developed on detached tubers when incubated in pots with plants than on those in boxes without plant material.

The higher incidence of black scurf after chemical haulm destruction than after haulm pulling may be due to increased exudation of nutrients, water and factors caused by stress. The increased formation of sclerotia after tissue damage or nutrient supply to the agar medium is in agreement with this view. The results from in vitro tests suggest that slight increases in exudation of water might facilitate uptake of nutrients and thus

enhance black scurf, but no evidence for such a relation with water was found in the field (Dijst, 1985; Dijst et al., 1986).

Stimulation of black scurf probably starts within 3 to 7 days after halum destruction. This can be inferred from the fact that a sudden increase in mycelium and black sclerotia appears on growing tubers between 7 and 10 days after haulm destruction but on agar plates a similar increase occurred within 4 days after a supply of nutrients. After COS, more black scurf developed than on untreated plants regardless whether tubers had been inoculated at 0, 9 or 13 days after the day of COS. Thus, no evidence was found for a short-term trigger to initiate the stimulation of black scurf formation after haulm destruction. Furthermore, this result indicates that an estimation of the inoculum density at the time of haulm destruction does not allow a prediction of black scurf development.

Haulm killing probably affects two different processes. First, initiation of sclerotia may be stimulated by an increase in the amount of a 'hyphal extension inhibitor', a fungal product reported to repress hyphal extension but not the total hyphal growth (Shibata et al., 1980b) or cAMP (Hashiba and Ishikawa, 1978). Secondly, haulm killing may increase the availability of nutrients or stress components which stimulate the growth and maturation of sclerotia. The second process is suggested by the experiments in vitro with nutrient supplements and in vivo by wounding of tubers.

Hyphal extension and hyphal growth are two separate processes influenced by different factors as appeared from studies with various carbon sources (Armentrout et al., 1986) and with validamycin on inhibition of hyphal extension (Shibata et al., 1980a). From these reports it may be concluded that tests with nutrients may serve to study sclerotial growth, but are useless to investigate sclerotial initiation.

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Samenvatting

Vorming van sclerotiën door Rhizoctonia solani op kunstmatige voedingsbodems en op aardappelknollen

Op voedingsbodems werd de sclerotiënvorming door *Rhizoctonia solani* Kühn AG-3 niet beïnvloed door een beperking van de hyfengroei. Wegnemen of toedienen van een voedingsbron verminderde resp. vermeerderde de totale myceliumgroei en sclerotiumvorming en de mate van vertakking van de hyfen, maar had geen effect op het tempo van initiatie en afrijping van de sclerotiën. De vorming van sclerotiën houdt dus geen verband met het afsterven van de kolonie. Het pas later toedienen van voeding aan mycelium gaf een ander verspreidingspatroon van de sclerotiën dan een continu voedingsaanbod.

Bij jonge groeiende knollen lijken, vooral bij de lenticellen, lakschurft-remmende factoren aanwezig te zijn. Op afrijpende knollen lijkt de sclerotiënvorming vergemakkelijkt te worden doordat die remming afneemt en door stimulerende factoren. Vervolgens lijken vluchtige exsudaten van ondergrondse delen van de plant die toename in lakschurft verder te bevorderen.

Vanaf zeven tot tien dagen na loofvernietiging is een versnelde toename van hyfen

en sclerotiën op de knollen zichtbaar. In een myceliummat op wateragar ontstond een dergelijke snelle toename binnen vier dagen na een voedselgift. Dit doet vermoeden, dat stimulering van lakschurft op knollen binnen drie tot zeven dagen na loofdoding begint. De stimulering lijkt niet in gang te worden gezet door een kort signaal of schilverruwing, maar lijkt te berusten op een samenspel tussen verschillende veranderingen: afname in remmende factoren en toename in exsudatie van voedingscomponenten, water en/of stressfactoren. Stressfactoren zouden van invloed kunnen zijn, want na verwonding van knollen nam de sclerotiënvorming toe. Exsudatie van water en nutriënten kunnen mede van invloed zijn gezien de sterkere sclerotiënvorming op vloeibare dan op agarbodems en na een extra voedingsgift. De resultaten geven aan dat de ontwikkeling van lakschurft na loofdoding niet kan worden voorspeld uit de lakschurftindex of inoculumdichtheid op de dag waarop het loof gedood wordt.

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