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Factors Affecting Translocation and Sclerotial Formation in *Morchella esculenta*

RACHEL AMIR,* DAN LEVANON,† YITZHAK HADAR,‡ AND ILAN CHET‡

**Migal—Galilee Technological Center, Kiryat Shmona 10200, Israel; †Institute of Soils and Water, Volcani Center, Bet Dagan, Israel; and ‡Otto Warburg Center for Agricultural Biotechnology, The Hebrew University of Jerusalem, Rehovot 76100, Israel*

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AMIR, R., LEVANON, D., HADAR, Y., AND CHET, I. 1995. Factors affecting translocation and sclerotial formation in *Morchella esculenta*. *Experimental Mycology* 19, 61–70. *Morchella esculenta* was grown on square split plates, forming sclerotia on one side and mycelium on the other. After the fungus ceased to colonize and before sclerotial initials appeared, [^{14}C]3-*O*-methyl glucose was added to the edge of the plate on the mycelial side. The effect of various activities in the mycelium (source) and sclerotia (sink) on sclerotial formation and translocation were examined using inhibitors and water potential changes of the media. Sodium azide or cycloheximide applied separately to both sides inhibited both sclerotial formation and translocation, showing that processes in the source and sink depend on metabolic activities as well as protein synthesis. The use of nikkomycin inhibited sclerotial formation, without affecting translocation to the sclerotia. Since the hyphal tips swelled and burst, the translocated compounds were lost to the media. In a strain defective in sclerotial formation, used as a control, no translocation took place, showing that there is a connection between sclerotial formation and translocation. Reversal of the water potential gradient between the two media (lower on the mycelial side), reduced the formation of sclerotia and translocation to them. Translocation to *Morchella* sclerotia takes place via turgor driven mass flow, but is nevertheless affected by activities in both the source and the sink.

INDEX DESCRIPTORS: translocation; *Morchella;* sink/source; sclerotia.

The edible mushroom *Morchella esculenta* Pers. forms sclerotia, whose importance in its life cycle was demonstrated by Ower *et al.* (1986). They found that fruiting bodies can be produced from sclerotia under controlled conditions. According to their findings, one of the critical requirements for the formation of sclerotia is their separation from the mycelium. The split-plate method developed by Amir *et al.* (1992) provided such separation, via differences in nutrient content and water potential between the media in the two compartments. One side of the plate contained Noble agar amended with 0.5 *M* glucose (GNA),[1] on which the sclerotia formed, and the other contained a defined medium (DM), on which the mycelia formed. Inoculum was placed in the GNA side, from which the hyphae grew towards the DM side. When the mycelial front reached the end of the DM side, four stages of sclerotial development were identified (initial, white, pigmented, and mature sclerotia). Amir *et al.* (1993) showed that during the formation of sclerotia, an increase in their biomass was accompanied by a decrease in mycelial biomass, demonstrating a relationship between sink and source and vigorous translocations towards the developing sclerotia.

Quantitative measurements of radioactive [^{14}C]glucose or its analog, [^{14}C]3-*O*-methyl glucose (Amir *et al.*, 1994), suggest that translocation to sclerotia occurs via turgor-driven mass flow, as in other fungi (Brownlee and Jennings, 1982; Granlund *et al.*, 1985; Jennings, 1987; Thain and Girvin, 1987). Although translocation is passive, it

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[1] Abbreviations used: GNA, glucose Noble agar; DM, defined medium; PDA, potato dextrose agar; [^{14}C]3-*O*-MG, 3-*O*-(^{14}C-methyl)-D-glucose.

is dependent on a variety of activities that take place in the hyphae of the source (loading) and sink (unloading). Previous research in other fungi has dealt only with source activity. Thus, Brownlee and Jennings (1982) added either sodium azide or 2 *M* glucose to the loading mycelium of *Serpula lacrimans* and found that the velocity of translocation decreased. Similarly, Brownlee and Jennings (1981) and Coggins *et al.* (1980) reported that the volume of droplets produced at the mycelial front in the same fungus decreased.

The aim of the present study was to gain a better understanding of the processes taking place during sclerotial formation in the source (mycelium) and sink (sclerotia) of *M. esculenta* and their influence on translocation. To this end, various glucose concentrations (changing the water potential of the media) and a number of inhibitors were studied.

MATERIALS AND METHODS

Experimental Setup

"Translocation plates" (Amir *et al.*, 1994), with some modifications were used. Square plates were divided in half with a 4-mm polycarbonate barrier to prevent diffusion. Twenty milliliters of Noble agar (Difco), supplemented with 90 g liter^{-1} glucose (GNA), was placed in one compartment, and 20 ml of DM was placed in the other (Amir *et al.*, 1994). Each compartment was covered with cellophane which had previously been boiled in water for 10 min and autoclaved. Five 4-mm disks of *M. esculenta* (from Fungi Perfecti; Olympia, WA) from potato dextrose agar (PDA) culture were transferred in a straight line to the edge of the plate on the GNA side, in order to achieve a uniform front of colony growth. As a control, strain ATCC 32785, which is defective in sclerotial formation, was studied in the same system. The plates were incubated at 25°C until the hyphae reached the opposite end of the plate. 3-*O*-(^{14}C-methyl)-D-glucose ([^{14}C]3-*O*-MG) in 100 μl of water (5 × 10^4 dpm; Sigma) was then carefully dripped along the edge of the plate on the DM side. Specific activity was 28 mCi mmol^{-1}. Each experiment was performed twice, with five replicates.

Measurement of Translocation

Four 1-cm wide strips of colonized agar were cut from each side of the plate, perpendicular to the direction of growth. The fungus was removed from the cellophane and its fresh weight (FW) was determined. The levels of radioactivity in the mycelium and agar strips were analyzed separately, by placing them in vials and running them through a liquid scintillation counter (Packard Tri-Carb, Model 4530) after 24 h in 4 ml scintillation liquid (Ultima-Gold, Packard).

Application of Inhibitors

At the end of colonization, when sclerotial initials began to form, the cellophane which separated the fungus from the medium was carefully removed and 100 μl of inhibitor (Table 1) was spread on the medium. The cellophane with the fungus was then replaced.

After 3 h, radiolabeled [^{14}C]3-*O*-MG was placed at the edge of the DM compartment, and 24 h later the internal structure of the hyphae was examined by light microscope (Zeiss, Model MC63). The amount of translocated radiolabeled material was analyzed by scintillation counter, 72 h after the application of [^{14}C]3-*O*-MG. The viability of the fungus on both sides of the plate was tested by transferring it to PDA and comparing its rate of growth to that of an untreated control.

RESULTS

Changes in Distribution of [^{14}C]*3-O-MG*

Radioactivity in the medium and in the fungus was measured at four stages of scle-

TABLE 1
Inhibitors Used in This Study, Source, and Activity

Inhibitor[a]	Source	Concn	Inhibitive activity	References
Sodium azide	Sigma	50 m*M*	Respiration	Brownlee and Jennings, 1982; Granlund *et al.*, 1985
Cycloheximide	Sigma	50 μ*M*	Protein synthesis	Hunter *et al.*, 1973; Okon *et al.*, 1973
Nikkomycin	Bayer	50 μ*M*	Chitin synthesis	Yarden and Yanofsky, 1991; Zhu and Gooday, 1992
Benomyl	Du pont	69 m*M*	Microtubule formation	Temperli *et al.*, 1991; Jochova *et al.*, 1993
Griseofulvin	Sigma	1 m*M*	Microtubule formation	Barja *et al.*, 1992
Colchicine	Sigma	2 m*M*	Microtubule formation	Brownlee and Jennings, 1981; Young, 1991
Cytochalasin B	Sigma	1 m*M*	Microtubule formation	Allen *et al.*, 1980; Cooper and Tinker, 1981
Ortovanadate	Sigma	1 m*M*	Membrane sugar carrier	Garril and Jennings, 1991
*p*CMBS	Sigma	2 m*M*	Membrane sugar carrier	M'Batchi *et al.*, 1985

[a] Inhibitor (100 μl) were applied to the appropriate compartment.

rotial development: (I) 24 h after application of the radioactive compound, when initials formed; (II) after 48 h, when white sclerotia formed; (III) after 72 h, when the sclerotia began to form yellow pigments; (IV) after 144 h, at maturity (Fig. 1).

As previously reported (Amir *et al.*, 1994), the most noteworthy phenomenon was the loss of radioactivity from the fungus to the supporting medium on both sides of the plate, during all stages. Up to 37% of the total radioactivity was found in the mycelium. During the first two stages, the [^{14}C]3-*O*-MG was found at the site of application. However, at the later stages it was found largely in the medium on the GNA side, especially in the first strip. By 144 h, the [^{14}C]3-*O*-MG had been readsorbed by the hyphae on the GNA side (Fig. 1b).

This experiment showed that the proportion of labeled material on the GNA side peaked at 72 h, representing 73% of the total material. Once this was established, further examinations were conducted only after 72 h.

The loss of radioactivity to the agar could be explained by passive exchange of the labeled sugar with glucose from the GNA, as well as by an active unloading process, as

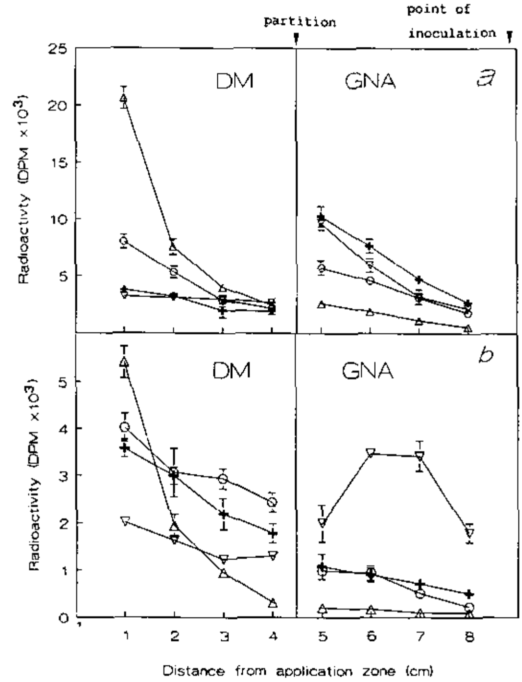

FIG. 1. Changes in radioactivity during the four stages of sclerotial development in *M. esculenta* (a) in the agar, (b) in the hyphae. Plates were analyzed 24 h (△), 48 h (○), 72 h (+), and 144 h (▽) after [^{14}C]3-*O*-methyl glucose was applied to the edge of the colony. Each number represents the total radioactivity of a 1-cm wide strip in agar and hyphae. Results are means of five replicates ±SD.

was demonstrated in *Claviceps* spp. by Mower and Hancock (1975). To elucidate this point, the glucose in the GNA compartment was replaced by mannose or fructose at the same concentration, yielding the same water potential. In neither case was the production of sclerotia affected. By following the fate of [^{14}C]3-*O*-MG, it was observed that (i) the rate of translocation was similar when glucose and mannose were applied, but lower with fructose; (ii) even though the sugar in the agar was not glucose, the greater part of the radioactivity (51–78%) was again lost to the medium (Fig. 2).

Effect of Glucose Concentration in the Media

The water potentials of the media in the plate, before inoculation, were −2.1 MPa (GNA side) and −0.5 MPa (DM side). Two experiments were conducted to test whether reversal of the water potential would affect sclerotial formation, i.e., when it was lower on the DM than on the GNA side. In the first experiment, no glucose was added to the GNA side, resulting in water potentials of −0.09 MPa (GNA), while the DM side remained unchanged. In the second experiment, in addition to the regular quantity of glucose added to the GNA compartment, 2 *M* glucose was added to the DM compartment, resulting in values of −2.1 MPa (GNA) and −7.2 MPa (DM).

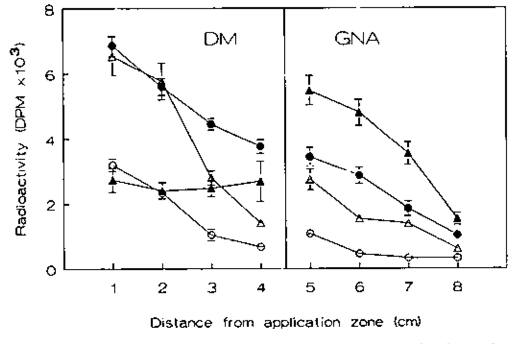

FIG. 2. Effect of fructose or mannose replacing glucose in the GNA compartment. Fructose applied (●, agar; ○, mycelium), mannose applied (▲, agar; △, mycelium).

The absence of glucose on the GNA side strongly reduced sclerotial formation, resulting in reduced translocation to that side (Table 2, Fig. 3). A similar effect on sclerotial formation was obtained in the second experiment. Most of the [^{14}C]3-*O*-MG (85%) was found on the DM side, though the formation of a few sclerotia on the GNA side led to some translocation to them. A low rate of uptake to the hyphae was observed (3.8%) in both compartments.

Translocation in a Defective Strain

In strain ATCC 32785, defective in sclerotial formation, hyphal branching and the width of the hyphae resemble those of the strain used in the present experiments, which does form sclerotia (Amir *et al.*, 1993). However, in ATCC 32785, the branches do not form initials. Biomass production is low, being 75% (dry wt) of that of the sclerotia-producing strain in the DM compartment, 4.5% in the GNA compartment. Similarly, although cytoplasmic movement was clearly seen in the hyphae in both directions, that movement was slower in ATCC 32785 than in the regular strain.

Translocation was measured 72 h after [^{14}C]3-*O*-MG had been placed at the limit of colonization. Only 2% of the total radioactivity was found in the mycelium (Fig. 4; Table 2). There was also a marked decrease in radioactivity with distance from the inoculation point of [^{14}C]3-*O*-MG in the DM compartment, possibly due to a wick effect (i.e., a stream on the surface of the hyphae). Thus, 60% of the total dpm was found in the first strip of agar, and no translocation to the GNA side was observed. A deficiency in the loading process appears to be the cause, since there was almost no uptake by the mycelium in the DM compartment.

TABLE 2
Translocation of (^{14}C)3-O-methyl Glucose in *M. esculenta* under Different Conditions

		Distance from application (cm)							
		DM				GNA			
Factor		1	2	3	4	5	6	7	8
Control	Agar	21	19	10	5	20	14	6	4
	Mycelium	20	17	12	10	5	3	2	2
Azide GNA side	Agar	93	46	37	13	0	0	0	0
	Mycelium	47	28	12	4	0	0	0	0
Azide DM side	Agar	3907	191	53	14	0	0	0	0
	Mycelium	0	5	2	1	0	0	0	0
Cyclohaximide GNA side	Agar	70	34	28	25	11	18	12	5
	Mycelium	49	24	25	22	1	2	2	1
Cycloheximide DM side	Agar	174	126	43	20	0	0	0	0
	Mycelium	9	6	5	2	0	0	0	0
Nikkomycin	Agar	62	27	16	16	22	120	263	217
	Mycelium	85	52	25	23	3	1	1	0
No glucose CNA side	Agar	33	28	12	7	11	4	5	6
	Mycelium	11	10	8	6	1	1	2	1
2M glucose DM side	Agar	108	77	93	34	10	14	19	13
	Mycelium	2	3	2	2	1	1	1	1
ATCC 32785	Agar	2899	377	117	64	2	1	0	0
	Mycelium	48	4	1	1	0	0	0	0

Note. Data are presented in terms of specific activity (DPM g · FW^{-1}). The radioactivity value in each strip, both in the hyphae and agar, was divided by the corresponding biomass value. The analysis was performed 72 h after application of [^{14}C]3-*O*-methyl glucose.

Effect of Azide and Cycloheximide

The effect of the respiratory uncoupler sodium azide (50 m*M*) was studied when applied separately to the two sides (Fig. 5; Table 2). Microscopic examination after 24 h showed that the addition of azide affected only the side to which it had been applied, destroying the internal structure of most of the hyphae. The viability of hyphae on the treated side was maintained, despite the damage; after 3 days, their development on PDA was equal to that of a control. When azide was applied to the DM side, the mycelium did not absorb [^{14}C]3-*O*-MG (a wick effect being observed instead), and the [^{14}C]3-*O*-MG therefore did not reach the GNA side (Fig. 5). When azide was applied to the GNA side, normal absorption was observed in the mycelium on the DM side, but translocation to the GNA side did not take place. This indicates that there must be metabolic activity in both the source and sink for translocation to occur.

The effect of adding cycloheximide (50 μ*M*) resembled that of adding azide, with some minor differences (Table 2). No damage to the hyphae was observed, and application of cycloheximide to the GNA side resulted in the absorption of [^{14}C]3-*O*-MG by the hyphae on the DM side, with some translocation to the GNA side (up to 4.4%). Application of cycloheximide to the DM side inhibited the loading process: only 6% of the total radioactivity was found in the mycelium on that side. Apart from these effects, sclerotial formation and translocation were inhibited as with azide.

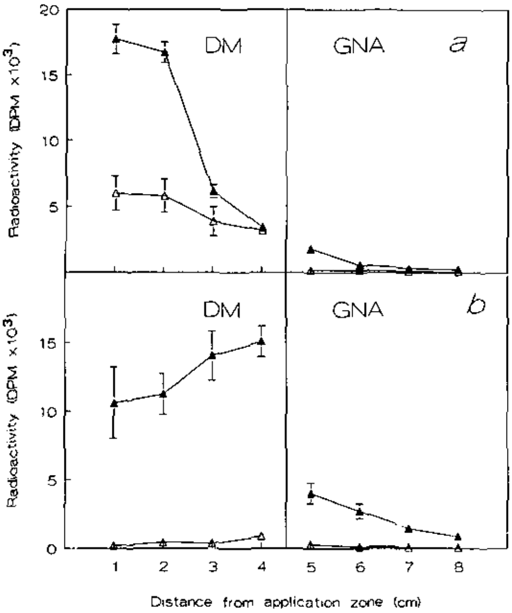

FIG. 3. Effect on translocation of [^{14}C]3-*O*-methyl glucose in *M. esculenta* of reversing the water potential gradient, by (a) omitting glucose in the GNA compartment or (b) adding 2 *M* glucose to the DM compartment (▲, agar; △, mycelium). Results are means of five replicates ±SD.

Effect of Nikkomycin

Nikkomycin is an antibiotic that inhibits chitin synthesis and cell wall biosynthesis, arresting hyphal growth and making the hyphal tips swell and burst (Zhu and Gooday, 1992). Nikkomycin was placed on the GNA side, at a concentration of 10^{-5} *M*. Its influence was observed particularly at the hyphal tips which characterized the sclerotial initials. Although the tips swelled and burst (Fig. 6), the fungus remained viable. Similarly, although sclerotial formation was inhibited (except for a few sclerotia that formed on the first strip), translocation to the GNA side was maintained (Table 2, Fig. 7). Calculation of the result on a fresh weight basis (Table 2) shows that there was high specific translocation to the GNA side, even though sclerotia were not formed.

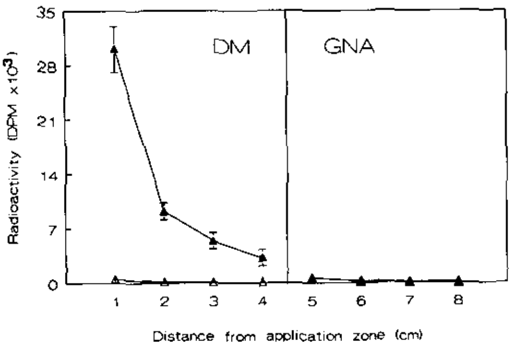

FIG. 4. Translocation of [^{14}C]3-*O*-methyl glucose in a strain of *M. esculenta* that does not form sclerotia (▲, agar; △, mycelium). Results are means of five replicates ±SD.

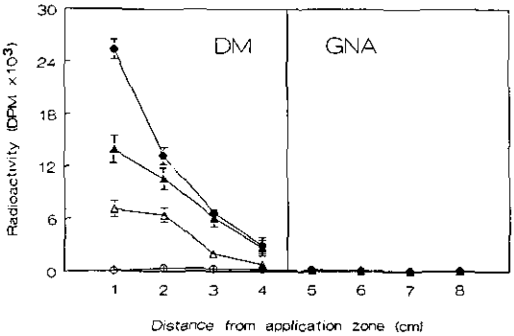

FIG. 5. Effect of sodium azide on translocations of [^{14}C]3-*O*-methyl glucose in *M. esculenta* applied to the GNA compartment (▲, agar; △, mycelium); applied to the DM compartment (●, agar; ○, mycelium). Results are means of five replicates ±SD.

Effects of Other Inhibitors

Applications of benomyl, griseofulvin, colchicine, and cytochalasin B have been reported to disrupt microtubule organization and contractile microfilaments in fungi and plants (Table 1). In the present experiments, however, no significant effect on sclerotial formation and translocation was observed. The addition of sodium orthovanadate or *p*-chloromercuribenzene sulfonic acid, both of which are known to inhibit sugar carriers on the plasmalemma of plants, did not inhibit the formation of sclerotia in this case. No plasmolysis was observed with any of these inhibitors. It was concluded that *Morchella* is not sensitive to these compounds. It is possible that they

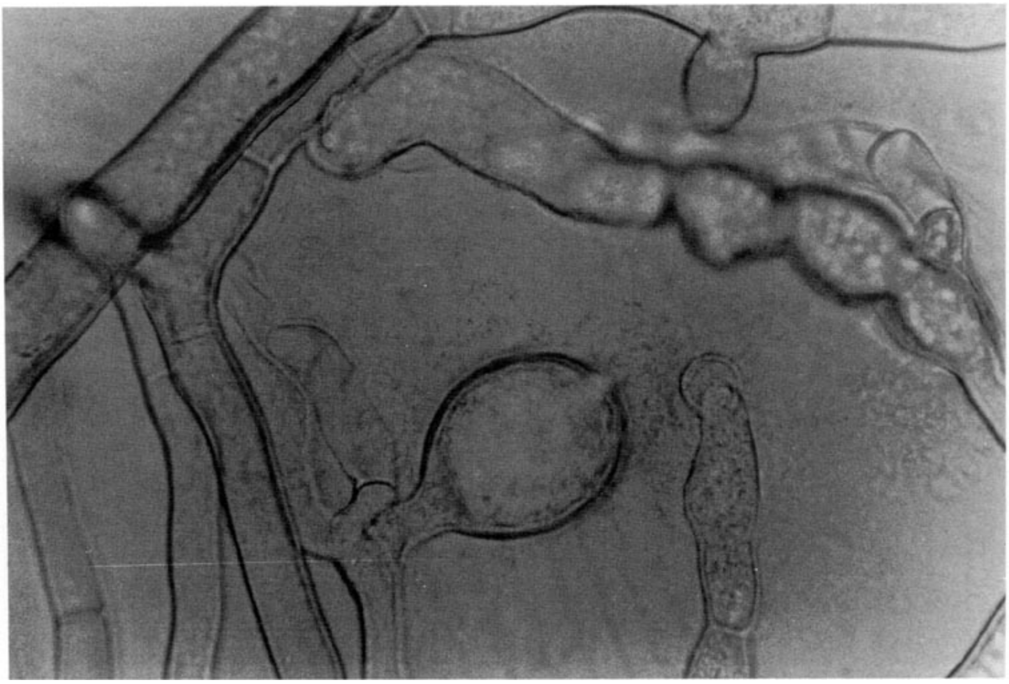

FIG. 6. Effect of 10^{-5} *M* nikkomycin on hyphae of *M. esculenta*. Swelling and bursting of hyphal tips.

did not penetrate the hyphae, or that they penetrated but caused no measurable effect.

DISCUSSION

Translocation of radioactive glucose and 3-*O*-MG took place during sclerotial formation. The velocity of translocation was much greater than the rate of utilization of nutrients in the developing sclerotia (Amir *et al.*, 1994). Most of the translocated labeled compounds (up to 80% of the total radioactivity in the GNA compartment) were lost to the medium. This phenomenon is thought to originate from excess nutrients which were not converted to insoluble reserves. This loss reduced the turgor potential in the GNA compartment. In addition to this process, a passive exchange between the translocated sugar and glucose in the medium takes place. To elucidate this point, mannose and fructose were tested as substrates in the GNA compartment. It is assumed that these two processes take place simultaneously.

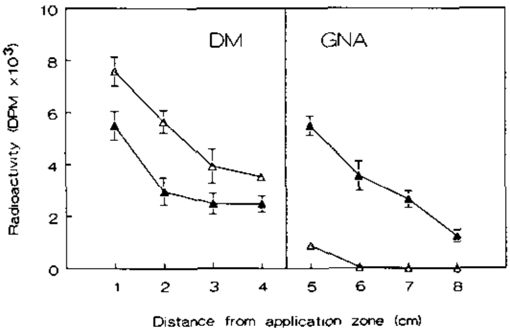

FIG. 7. Effect of nikkomycin on translocation of [^{14}C]3-*O*-methyl glucose in *M. esculenta* applied to the GNA compartment (▲, agar; △, mycelium). Results are means of five replicates ±SD.

For translocation to occur, various activities of the source and sink are required, which give rise to gradients of turgor pressure between the two compartments of the plate, thereby driving translocation. Consequently, a number of inhibitors and other factors which affect the loading and unloading processes were used to follow the process.

The general inhibitors, sodium azide and cycloheximide, were employed. Both inhibited translocation and sclerotial formation. Their application to the DM compartment inhibited loading, as well as the synthesis of cytoplasm, with the result that no gradient of osmotic potential between the mycelium and medium was created. Consequently, no water flowed into the mycelium and no turgor pressure was created to drive translocation. Application of these inhibitors to the GNA compartment demonstrated that the hydrostatic pressure created in the DM compartment is not sufficient, by itself, to cause translocation. Active unloading on the sclerotial side is also required to remove the solutes, i.e., to lower the turgor potential and thereby facilitate translocation. Brownlee and Jennings (1981, 1982) have shown that the use of sodium azide at the food source of *S. lacrimans* rhizomorphs inhibits translocation, as well as droplet production by hyphal tips. These authors showed that the droplets are produced by pressure-driven water flow in the hyphae at the sink. With regard to the application of cycloheximide, Okon *et al.* (1973) found that this inhibitor, applied to the colony margins in *Sclerotium rolfsii*, prevents protein synthesis, and hence sclerotial formation, without inhibiting translocation. They concluded that translocation and protein synthesis are independent processes, both of which are essential to sclerotial formation. In our experiments, cycloheximide also prevented sclerotial formation, but also inhibited translocation, implying interdependence.

Unlike the above inhibitors, whose effect is general, nikkomycin specifically inhibits the synthesis of chitin. Although this inhibited sclerotial formation, it did not affect translocation. Nikkomycin caused the cells of the hyphal tips to swell and burst, releasing translocated material—including radioactive material—into the medium. In other cells, it is likely that some of the processes associated with translocation at the sink were maintained, despite the application of nikkomycin, causing loss from the hyphae and inhibiting respiration and intracellular storage. Such activity would lower solute concentration in the hyphae, and hence turgor potential, enabling translocation to take place.

Both the translocation process and sclerotial formation require a water potential gradient, i.e., lower water potential in the GNA compartment. In both sets of experiments in our study, both processes were inhibited when this potential was reversed, i.e., was lower in the DM compartment. The high concentration of glucose normally found in the GNA compartment is used as a food base (Amir *et al.*, 1992), but it has an additional role in that it creates a low water potential, thereby increasing the flow of water from the developing sclerotia. This causes reduced turgor, thereby increasing the pressure gradient from source to sink, further increasing flow. This finding is in agreement with abundant evidence from the plant kingdom indicating that most, if not all, strong sinks have a high concentration of solutes in the apoplast (Lucas and Madore, 1988).

When 2 *M* glucose was supplied to the DM compartment, a low number of sclerotia still formed in the GNA compartment, even though the gradient was reversed. Since 14.5% of the total radioactivity was found in the GNA compartment, it appears that there was a turgor gradient in some hyphae, allowing sclerotia to be formed. This finding confirms those of Thompson *et al.* (1985), who reversed the water potential across the mycelium of *S. lacrimans* so that the turgor gradient would be higher at the mycelial front. Extension of the mycelial front continued, with positive turgor being maintained in the extending hyphae. These authors concluded that the water potential within the hyphae is protected from changes in water potential in the mycelium behind the front. On the other hand, in experiments by Brownlee and Jennings (1982), in which 2 *M* glucose was supplied to the mycelium at the food source, translocation ceased almost immediately.

The results of this study strengthen the hypothesis that translocation is brought about by a bulk flow mechanism. It is postulated that nutrients are loaded into the mycelial hyphae on the DM side, together with the synthesis of cytoplasm rich in solutes, thus lowering the water potential in the mycelium and attracting water into the hyphae. This process increases turgor, generating the pressure to drive water and solutes to the sclerotia. However, this pressure is not high enough to cause translocation and, consequently, additional strong unloading activity is required at the sink. In the sink, the solutes are utilized by conversion to insoluble forms and by exudation of the excess to the medium, thereby decreasing the turgor. In addition, the high concentration of glucose in the GNA compartment attracts the flow of water from the sclerotia and thereby encourages translocation.

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