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Effects of elevated substrate–ethylene on colonization of leek (*Allium porrum*) by the arbuscular mycorrhizal fungus *Glomus aggregatum*

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Abstract: There are very few studies of hormonal regulation of arbuscular mycorrhiza formation that include the gaseous hormone ethylene. Ethylene is considered inhibitory to the formation of arbuscular mycorrhizae; however, very low concentrations may promote their formation. We used an improved method of exogenous ethylene application to determine whether ethylene concentration dependent changes in colonization occur in the leek (*Allium porrum* L. cv. Giant Musselburgh) – *Glomus aggregatum* Schenck & Smith emend. Koske system. This improved method allowed for a continuous flow of constant concentration of the gas to be applied to a substrate. The 0.6 µL/L substrate–ethylene treatment reduced both root and leaf length and resulted in significantly lower arbuscular colonization compared with controls, whereas the 0.3 µL/L treatment reduced root length only and did not significantly affect colonization levels. Despite continuous application of exogenous ethylene, the amount of ethylene detected in inoculated substrates was reduced to near zero 20 days after inoculation. This decrease may be either due to an increased capacity for ethylene oxidation by arbuscular mycorrhizal roots or because arbuscular mycorrhizal fungi (or other microbes in the pot-cultured inoculum) are capable of metabolizing ethylene. The present study highlights the need for investigations into arbuscular mycorrhizal fungal physiology and the mechanisms by which ethylene regulates arbuscular mycorrhiza formation.

Key words: arbuscular mycorrhiza, colonization, exogenous ethylene, monocot.

Résumé : Très peu de travaux faits sur la régulation hormonale du développement des endomycorhizes ont inclus l'effet de l'éthylène. L'éthylène est reconnu comme un inhibiteur de la formation des mycorhizes; cependant, une concentration basse est supposée être stimulatrice. Nous avons utilisé une méthode d'application exogène, afin de déterminer si la colonisation du poireau (*Allium porrum* L. cv. Giant Musselburgh) par le champignon endomycorhizien *Glomus aggregatum* Schenck & Smith emend. Koske est dépendante de la concentration du gaz. Par cette méthode, l'éthylène est distribué à une concentration constante dans le sol et d'une manière continue. Le traitement à 0,6 µL/L inhibe la croissance racinaire et celle de la tige et décroît la colonisation arbusculaire, alors qu'une concentration de 0,3 µL/L affecte la croissance racinaire seulement, mais pas le niveau de colonisation. Cette réduction peut être expliquée soit par une plus forte capacité de la part des racines mycorhiziennes à oxider l'éthylène soit par une capacité de la part des champignons endomycorhiziens à métaboliser l'éthylène. Notre travail accentue le besoin d'études sur la physiologie de ces champignons et sur les mécanismes par lesquels l'éthylène contrôle la formation d'endomycorhizes.

Mots clés : mycorhizes à arbuscules, colonization, application exogène d'éthylène, monocotylédones.

Introduction

Despite growing evidence for the involvement of phytohormones in the formation of arbuscular mycorrhizae (AM), very few studies have considered the effects of ethylene on this symbiosis (Ludwig-Müller 2000). To date, all studies of ethylene effects on AM formation, except one, suggest that this phytohormone is inhibitory to AM formation (Azcon-Aguilar et al. 1981; Morandi 1989; Ishii et al. 1996; Geil et al. 2001). Only Ishii et al. (1996), using the trifoliate orange (*Poncirus trifoliata*) – *Gigaspora ramisporophora* system, have demonstrated that ethylene may either inhibit or stimulate AM formation depending on the concentration applied; interestingly, stimulation occurred as a result of applying the extremely low concentration of 0.05 µL/L.

Most previous studies have employed methods that do not allow for the sustained application of an accurate concentration of ethylene. For example, Azcon-Aguilar et al. (1981) and Morandi (1989) used ethrel (an ethylene-releasing chemical) as a source of ethylene; this technique does not allow for the application of a constant concentration of the gas, and the amount of ethylene liberated from the ethrel treatments was not determined in either study. Additionally, the hydrolysis of ethrel results in the formation of phosphate as a by-product (Abeles et al. 1992), which complicates AM

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DOI: 10.1139/B01-135

studies, since increased availability of phosphorus can limit colonization by AM fungi (Smith and Read 1997). Ishii et al. (1996), although using ethylene gas instead of ethrel, only applied a small volume (2 L) of an ethylene–air mixture to treatment substrates once per day. Using this method over the course of an experiment, the actual concentration of ethylene in the treatment substrate is not equal to the concentration of gas applied.

The objective of this study was to use an improved method of applying exogenous ethylene (one that allows for continuous application of a constant, known concentration of the gas) to determine if ethylene concentration dependent alterations in colonization by *Glomus aggregatum* Schenck & Smith emend. Koske can be induced in leek (*Allium porrum* L. cv. Giant Musselburgh). Two concentrations of ethylene gas that are physiologically relevant in nonstressed environments (i.e., <1 µL/L) were tested under the hypothesis that one (0.6 µL/L) would inhibit colonization and the other (0.3 µL/L) would stimulate colonization. These two arbitrary concentrations were chosen based on the suggestion by Feldman (1984) that, in nonstressed environments, a regulatory role for ethylene in plant growth and development is likely at levels below 0.5 µL/L.

Materials and methods

Seeds of leek were surface sterilized in an aqueous solution of 8% household bleach (5.25% sodium hypochlorite) for 5 min, rinsed three times in deionized water (1 min each), and left to imbibe overnight in a fresh rinse of deionized water. Imbibed seeds were placed on moist filter paper in Petri plates for 1 week and then planted in sterile Turface® (Applied Industrial Materials Corp., Buffalo Grove, Ill.) saturated with water. Potted seedlings were placed in miniature hothouses (Guinel and Sloetjes 2000), which were kept in a growth-room (16 h light : 8 h dark cycle at a temperature of 23:18°C (light:dark)) under incandescent and cool white fluorescent lamps with a total light intensity of 240 µmol·m^{-2}·s^{-1} photosynthetic photon flux (PPF). After 1 week, plants were removed from the miniature hothouses and watered as needed for an additional week.

At this time, seedlings were transplanted into Conetainers™ (560 mL volume Ray Leach Conetainers™, Stuewe & Sons Inc., Corvallis, Oreg.) containing autoclaved substrate with or without pot-cultured inoculum of the AM fungus (13 parts substrate to 1 part inoculum, v/v). The substrate consisted of Turface® and peat (Greenworld Garden Products, Pointe Sapin, N.B.) mixed in a 3:1 (v/v) ratio. The inoculum consisted of desiccated leek roots, previously colonized by *G. aggregatum*, cut into fine fragments, which were mixed in a Turface® carrier.

Exogenous ethylene was applied using the apparatus of Lee and LaRue (1992*a*, 1992*b*) with some modifications (see Geil et al. 2001). Prepared Conetainers™ were racked into each of four chambers in such a way that the shoots of all plants were in the same ambient atmosphere of the growth-room, whereas the roots (in their substrates) were subjected to various air and (or) ethylene treatments. One chamber (static control) received neither air nor ethylene. Another chamber (air-only control) received no ethylene but

did receive 2 L/min air. Each of the remaining two chambers received one of two concentrations of ethylene mixed in air (flow rate of 2 L/min); one chamber received a continuous flow (mean ± SD) of 0.34 ± 0.03 µL/L ethylene and the other 0.60 ± 0.01 µL/L ethylene.

Four replicate experiments were performed at separate times. For each replicate experiment, six Conetainers™ (three inoculated and three noninoculated) were racked into each treatment chamber; each Conetainer™ contained three seedlings. At harvest, measurements of growth characteristics were taken from all three seedlings in each Conetainer™ and the mean of these measurements was used as the value for a given Conetainer™. The three root systems from one Conetainer™ were then pooled and a subsample for quantification of colonization was taken from this pool. Thus, three values for colonization were obtained per treatment per replicate experiment; the mean of these three values was used as the colonization value for a given treatment of a given replicate.

The apparatus was kept in the growth-room described above where the seedlings were subjected to 27 µmol·m^{-2}·s^{-1} PPF (fluorescent lights only) for the first 3 days after transplanting, after which they were exposed to 400–520 µmol·m^{-2}·s^{-1} PPF (fluorescent lights and sodium halide lamps) until root collection and processing 28 days after transplanting. Each Conetainer™ received 30 mL of water approximately every 3 days. Low-phosphate (54.4 mg·L^{-1} KH$_2$PO$_4$) nutrient solution was applied instead of water on the third and nineteenth days after transplanting; this solution was prepared as described in Geil et al. (2001) except 27.0 mg·L^{-1} FeCl$_3$·6H$_2$O was substituted for 82.0 mg·L^{-1} Fe(III) EDTA.

Syringe air samples (1 mL each) were collected at least 5 days per week from the noninoculated and inoculated substrates from each of the four chambers; these samples were obtained from septum-capped lengths of PVC tubing inserted during Conetainer™ filling. On the same sampling days, 1-mL samples were also taken from the three chamber inflow lines. All samples were analyzed for ethylene by gas chromatography (Perkin-Elmer 3920 GC with a flame ionization detector; 1.83-m stainless steel column (0.3175 cm outside diameter) packed with Porapak N (80/100 mesh); He$_2$ carrier gas at 22 mL·min^{-1}; column temperature of 115°C). Ambient air samples were also collected and analyzed on each sampling day to ensure that the growth-room remained free of ethylene.

The number of adventitious roots and the length of the longest one, the number of leaves and the length of the longest one, and shoot dry weight were measured at harvest time, just before root processing. Data presented are mean values ± SE of four replicate experiments. Data were analyzed by two-way ANOVA followed by Tukey tests to determine the significance ($P < 0.05$) of treatment differences.

All adventitious roots were excised at the base of the shoot and processed according to Brundrett et al. (1994) with slight changes. They were fixed in 50% ethanol for a minimum of 24 h and cut into fragments approximately 1 cm in length. About 60 fragments were subsampled randomly (from the three root systems pooled from each Conetainer™) and cleared by autoclaving (120°C for 15 min) in 10% KOH (w/v).

Table 1. Mean values ± SE (four replicate experiments) of various characteristics measured on *Allium porrum* plants grown for 28 days in substrate with (I) or without (N) pot-cultured inoculum of *Glomus aggregatum* added and subjected to various ethylene and (or) air treatments.

Treatment	No. of adventitious roots	Longest adventitious root (mm)	No. of leaves	Longest leaf (mm)	Shoot dry mass (mg)
I static	4.50±0.09	244.35±12.53	2.22±0.12	179.45±13.09	18.03±1.17
I air only	4.64±0.36	260.63±8.90	2.23±0.08	183.95±8.35	16.95±1.53
I 0.3 µL/L	4.21±0.24	230.60±24.50	2.51±0.08	164.08±14.55	14.05±1.09
I 0.6 µL/L	4.58±0.31	207.48±16.81	2.23±0.08	131.78±13.60	11.50±1.93
N static	4.63±0.15	252.05±15.33	2.22±0.16	162.35±7.82	14.30±1.51
N air only	5.03±0.27	275.88±2.68	2.56±0.21	194.73±16.76	21.38±2.70
N 0.3 µL/L	5.15±0.27	220.05±12.37	2.51±0.05	165.93±9.44	17.68±1.50
N 0.6 µL/L	5.33±0.28	184.63±14.76	2.34±0.18	149.55±17.88	16.25±3.30
ANOVA summary					
I	**	ns	ns	ns	ns
E	ns	***	ns	*	ns
I × E	ns	ns	ns	ns	ns

Note: Static, no airflow or ethylene; air only, continuous flow of air (2 L/min); 0 3 µL/L, continuous flow of 0.3 µL/L ethylene in 2 L/min air; 0.6 µL/L, continuous flow of 0.6 µL/L ethylene in 2 L/min air. I, inoculum; E, ethylene treatment; I × E, interaction; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant.

Subsamples were rinsed in water after clearing and placed in 1% HCl (v/v) for 5 min. Root fragments were then placed directly into 0.01% acid fuchsin (Kormanik and McGraw 1984) and stained for 1 h at 55°C. Stained fragments were placed in 100% glycerol for a minimum of 24 h to destain. Subsequent to scoring, slides were examined using laser scanning confocal microscopy (see Geil et al. 2001) to obtain detailed observations of AM morphology.

Colonization levels were determined using the magnified intersections method (McGonigle et al. 1990). Forty-five of the 60 fragments from each root system subsample were mounted on glass slides and scanned at 312× total magnification using Nomarski optics on a Leitz Wetzlar compound light microscope (Plan ×25, NA 0.50 objective, 10× ocular with a crosshair). During scanning of slides, each intersection between a root fragment and the ocular crosshair was scored for the presence of at least one appressorium, intraradical hypha, and (or) arbuscule. Colonization values are expressed as a percentage of the total intersections scored (about 150 per subsample) and represent the mean ± SE of four replicate experiments. Since mean percent arbuscular colonization of plants from static treatment Conetainers™ of the first replicate differed significantly (Student *t* tests, $P < 0.05$) from those of the subsequent three replicates (data not shown), paired *t* tests (rather than ANOVA) were used to determine significant differences between treatments. All statistics were performed using SigmaStat® 2.03 software (SPSS Inc., Chicago, Ill.).

Results and discussion

Treatment effects on plant growth

The effects of the various combinations of inoculum and ethylene treatments on plant growth are shown in Table 1 along with a summary of the two-way ANOVA results. At the time of harvest, no obvious differences in root architecture (i.e., branching) or root diameter were observed between treatments. Inoculated plants did have 0.55 less adventitious roots than noninoculated plants (Tukey test, $P =$ 0.006), but inoculum did not have a significant effect on any other characteristic examined (Table 1).

Ethylene treatment significantly decreased the length of the longest adventitious root and the length of the longest leaf but did not affect any other characteristic measured (Table 1). Adventitious roots of plants from the 0.6 µL/L ethylene treatment were significantly shorter than those of plants from both the air-only and static controls (Tukey tests, $P <$ 0.001 and $P = 0.008$, respectively). Additionally, the adventitious roots of plants from the 0.3 µL/L ethylene treatment were significantly shorter than those of plants from the air-only control (Tukey test, $P = 0.036$). To our knowledge, no literature is available on the effects of ethylene on the growth and development of leek. However, our findings are consistent with those of studies of ethylene effects on root elongation in other monocots. For example, ethylene has been shown to inhibit root elongation in maize (*Zea mays*) (≥0.1 µL/L, Whalen and Feldman 1988), wheat (*Triticum aestivum*) (0.1–5 µL/L, Huang et al. 1997), barley (*Hordeum vulgare*), and rye (*Secale cereale*) (≥1 µL/L, Smith and Robertson 1971). However, root elongation may also be slightly promoted in rye (Smith and Robertson 1971) and in wheat (Huang et al. 1997) at very low ethylene concentrations (<1 and up to 0.1 µL/L, respectively). The longest leaves of plants from the 0.6 µL/L ethylene treatment were significantly shorter than those of plants from the air-only control treatment (Tukey test, $P = 0.006$). Similarly, Huang et al. (1997) reported limited shoot growth of wheat in response to 5 µL/L ethylene. There was no interaction between inoculum and ethylene treatments for any of the characteristics examined (ANOVA summary in Table 1). Interestingly, 0.6 µL/L substrate–ethylene is a high enough level to significantly influence the growth of leek roots and shoots, but 0.3 µL/L substrate–ethylene appears to affect roots only. This finding may suggest that leek roots are more sensitive to ethylene than shoots; however, the difference in root and shoot growth responses seems likely to be a result of applying ethylene to the substrate while the shoots remained in an open atmosphere.

Fig. 1. Ethylene measured in substrates containing leek roots without pot-cultured inoculum of *G. aggregatum* added from (*a*) 0.3 µL/L ethylene and (*b*) 0.6 µL/L ethylene treatments. Ethylene gas was applied in 2 L/min airflow. Different symbols represent different replicate experiments. Mean ± SD ethylene levels = 0.33 ± 0.005 in Fig. 1*a* and 0.59 ± 0.01 in Fig. 1*b*.
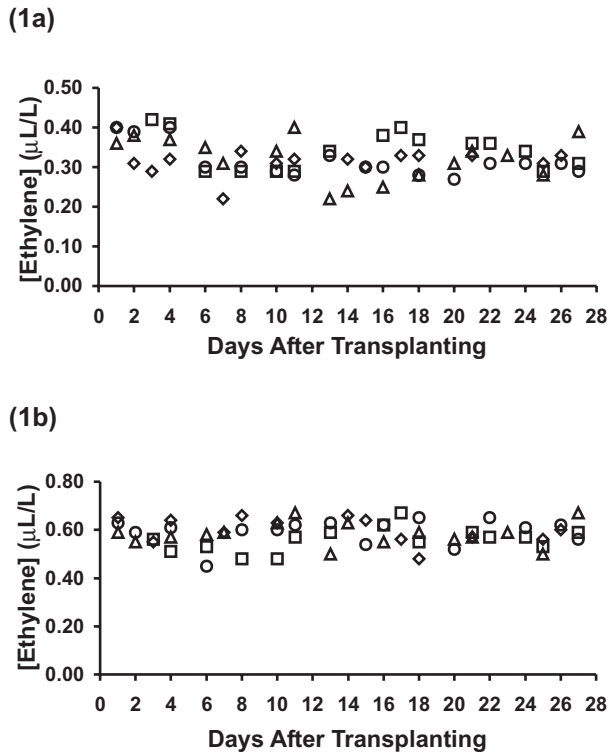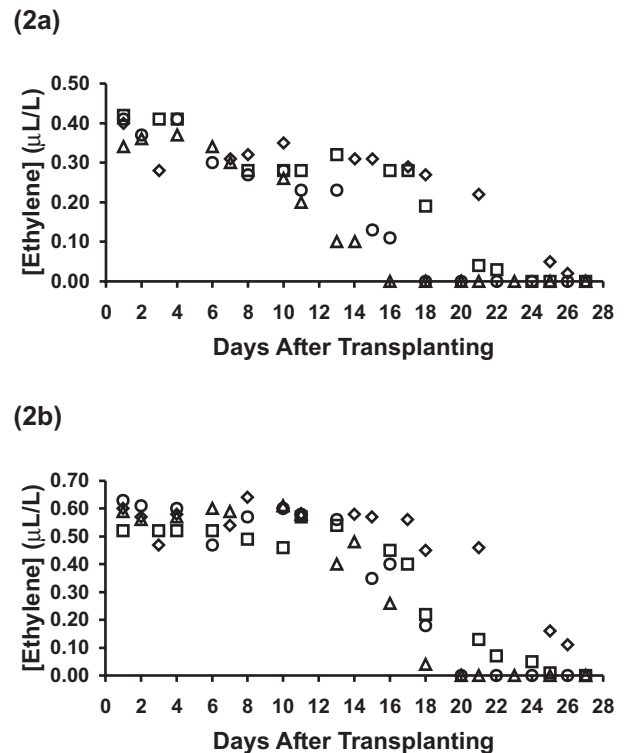
Fig. 2. Ethylene levels measured in substrates containing leek roots with pot-cultured inoculum of *G. aggregatum* added from (*a*) 0.3 µL/L ethylene and (*b*) 0.6 µL/L ethylene treatments. Ethylene gas was applied in 2 L/min airflow. Different symbols represent different replicate experiments.

(1a)

(2a)

(1b)

(2b)

Monitoring ethylene levels

Fluctuations in chamber inflow (not shown) and noninoculated substrate ethylene concentrations over the course of each experiment were minimal for both ethylene treatments (Fig. 1). Surprisingly, 14 days after transplanting, the concentration of ethylene in the inoculated substrates of each ethylene treatment began to decrease despite continuous application of constant concentrations of this gas; 20–27 days after transplantation, ethylene was no longer detectable in inoculated substrates (Fig. 2).

Roots alone are capable of oxidizing ethylene (Abeles 1984; Jackson 1991); however, if the decreases were due to root oxidation, similar decreases would have been observed in noninoculated substrates. McArthur and Knowles (1992) have demonstrated that AM fungal colonization of potato (*Solanum tuberosum*) results in decreased production of ethylene by roots via inhibition of the enzyme that converts the direct precursor 1-aminocyclopropane-1-carboxylic acid to ethylene. Subsequent reports support McArthur and Knowles' (1992) finding that the establishment of an AM symbiosis results in regulation of endogenous host ethylene production (Besmer and Koide 1999; Cruz et al. 2000). The fact that host ethylene biosynthesis is altered as a result of the formation of an AM association (e.g., McArthur and Knowles 1992) suggests that the decrease in exogenous ethylene observed in the present study may be due to an increased capacity of AM roots to oxidize ethylene.

Alternatively, either *G. aggregatum* or a foreign component(s) in the pot-cultured inoculum (or both) was responsible for metabolizing ethylene in the inoculated substrates. The presence of one or more non-AM fungi and (or) a variety of bacteria in pot cultures of AM fungi is highly likely (and nearly unavoidable). Although the pot cultures used in this study were not assessed for the presence of bacteria, non-AM fungal hyphae (particularly *Rhizoctonia* sp.) were observed during quantification of AM fungal colonization. Several species of fungi are known to be ethylene producers (Abeles et al. 1992); however, information regarding the metabolism of ethylene by fungi is lacking. Nonetheless, the possibility that AM fungi may be capable of metabolizing ethylene should be investigated further. In nature, the actinomycete *Mycobacterium paraffinicum* uses ethylene as a carbon source (Abeles 1984) and keeps ethylene levels in most soils near zero (Abeles et al. 1992).

Quantification of AM fungal colonization

When compared with the static control, the air treatment did not significantly affect the prevalence of any symbiotic fungal structures scored (Fig. 3). Similarly, the 0.3 µL/L ethylene treatment did not significantly affect the amount of fungal structures scored compared with the air-only control; in contrast, the 0.6 µL/L ethylene treatment significantly reduced the prevalence of both intraradical hyphae and arbuscules (Fig. 3). This decrease in colonization could be the result of 0.6 µL/L ethylene

causing a root-surface block and (or) limiting the extension of colonization units along the longitudinal axes of roots. These characteristics have been observed in pea AM treated with 5.5 µL/L ethylene, but it is not known if they are the result of ethylene affecting the AM fungus directly or indirectly via its effects on the host (Geil et al. 2001). The decrease in colonization observed in roots exposed to 0.6 µL/L ethylene is not a function of root penetration attempts, as no differences in the numbers of appressoria formed on roots were detected between treatments (Fig. 3). A root-surface or other spatial block to colonization was not detected in 0.6 µL/L ethylene treated roots; however, for accurate detection of the success of root entry, modification of the magnified intersections method is necessary (see Geil et al. 2001), and this method was not employed in the present study. Unlike AM fungal colonization in pea roots exposed to 5.5 µL/L substrate–ethylene (Geil et al. 2001), reduced colonization in leek (as a result of applying 0.6 µL/L substrate–ethylene) was not accompanied by morphological alterations at any stage of development. In fact, laser scanning confocal microscopy revealed that AM formed in all treatments were very similar in morphology to that described for *Glomus versiforme* in roots of leek (Lackie et al. 1987). There was not a significant difference in colonization between 0.3 µL/L ethylene treated roots and those from control treatments; however, 0.3 µL/L ethylene treated roots did have significantly more arbuscules than 0.6 µL/L ethylene treated roots.

Conclusions

Using a technique that allows for the continuous application of a constant, known concentration of ethylene, we have demonstrated that 0.6 µL/L substrate–ethylene significantly reduces colonization of leek by *G. aggregatum*. This finding supports previous reports of AM formation inhibition in a variety of host–fungus combinations by various concentrations of ethylene (Azcon-Aguilar et al. 1981; Morandi 1989; Ishii et al. 1996; Geil et al. 2001). Whereas Ishii et al. (1996) reported increased colonization at 0.05 µL/L ethylene, in this study, treatment with 0.3 µL/L ethylene did not significantly stimulate colonization compared with controls; however, this treatment did significantly increase arbuscular colonization compared with the 0.6 µL/L ethylene treatment.

The hypothesis that a component of the AM fungal pot-cultured inoculum was responsible for the consumption of exogenous ethylene warrants further investigation. As mentioned earlier, it is possible that *G. aggregatum* is capable of metabolizing ethylene; however, the present study could not rule out that bacteria or a non-AM fungal species was responsible for this observation or conversely that AM roots exhibited an increased capacity for ethylene oxidation.

Sensitivity to ethylene is known to vary greatly among plant species (Smith and Robertson 1971; Jackson 1991), and likely the same is true for fungal species. Thus, the role of a given level of ethylene probably changes depending on the ethylene sensitivities of the host plant and the AM fungus involved in a particular association.

Fig. 3. Colonization of leek roots by *G. aggregatum* 28 days after inoculation as determined by the magnified intersections method. The same letters over bars indicate significant differences (paired *t* test, $P < 0.1$ (*a* and *b*) and $P < 0.05$ (*c*)). Values represent the mean of four replicate experiments. Error bars indicate SE. Static, no air or ethylene; air only, air flow at 2 L/min. Ethylene was applied in 2 L/min airflow.
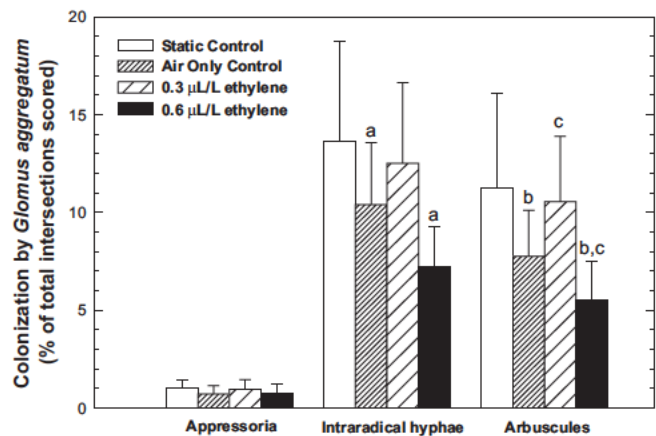
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