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# Endocellulase activity is associated with arbuscular mycorrhizal spread in pea symbiotic mutants but not with its ethylene content in root

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## Abstract

Plant cell wall hydrolytic enzymes seem to be important to root penetration by arbuscular mycorrhiza (AM) fungi and development of AM symbiosis. In this study, taking endocellulase activity as an enzymatic model, the possibility was tested that variations in fungal colonization due to different plant capacities to form AM, can be a good experimental system to identify hydrolytic enzymes which are important to root colonization. Quantitative and qualitative endocellulase activity in roots of different symbiotic pea mutants was analysed. There were differences in root colonization among plant mutants according to their symbiotic features and a similar behaviour in fungal colonization capacities and increases in endocellulase activity in roots was also found. Mutant E107 showed the highest ethylene quantity among the phenotypes analysed, and this phytohormone could be responsible for the decrease in colonization in the mutant, but did not have any effect on cellulase activity during mycorrhiza formation. Results suggest that changes in endocellulase activity in colonized roots are associated with fungal spread within the cortex and arbuscule formation.

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*Keywords:* Arbuscular mycorrhiza; Cellulase; Pea mycorrhizal mutants

## 1. Introduction

Root colonization by arbuscular mycorrhizal fungi (AMF) results in a positive effect on plant growth mainly because the nutritional status of the host plant is improved by transferring phosphate through the AM hyphae from the soil to the plant (Smith and Read, 1997). While enhanced plant mineral nutrition is of immense significance, other aspects are important in the ecology and physiology of AM plants, including a bioprotectional effect against soil-borne pathogenic fungi (recently reviewed by St-Arnaud and Vujanovic, 2006), and the alleviation of abiotic stresses (e.g. water deficiency, heavy metals contamination). In addition, the extraradical AM fungal hyphae affect positively the formation and stability of soil aggregates, improving soil fertility (Rilling et al., 2003). However, transfer of carbon to the biotrophic AMF is a cost incurred by the host plant as a consequence of infection, and in natural conditions it is often difficult to demonstrate the net benefit that infected plants receive from the association (Fitter, 1991). During the symbiotic stage, the AMF penetrated the root, formed its intraradical structures, e.g. inter and intraradical hyphae and arbuscules, and an exchange of nutrients between the host and the symbiont occurs.

The formation of the AM is a complex developmental event requiring the coordination of signal release and perception, gene expression and protein action of two distinct partner organisms. In this context, plant cell-wall components and plant cell-wall degrading enzymes could play a important role in plant-fungi interactions because the plant cell-wall may be important to fungi not only for penetration and ramification inside the plant tissue but also for obtaining nutrients (Radford et al., 1996). Plant pathogenic fungi synthesize and secrete large quantities of cell-wall-degrading enzymes to invade the plant tissue and their regulation has been extensively studied (Deising et al., 1995; Annis and Goodwin, 1997). On the other hand, there are cell-wall-degrading enzyme activities during the

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establishment of Mycorrhiza, and these enzymes seem to be involved in the penetration and development of both ectomycorrhizal (Cairney and Burke, 1994) and AM fungi in plant roots (García-Garrido et al., 2002).

Mycorrhizal fungi produce very small quantities of plant cell-wall-degrading enzymes (García-Romera et al., 1991; García-Garrido et al., 1992, 2000). Furthermore, the cell-wall-degrading enzymes produced in mycorrhizal roots show similar electrophoretic and biochemical characteristics to the plant enzymes produced in non-mycorrhizal roots (García-Romera et al., 1997; García-Garrido et al., 1996, 2000). This suggests that the role of fungal enzymes is only to selectively and specifically break plant cell-wall components for fungal penetration.

Among plant cell-wall polymers cellulose is the major structural polymer in primary cell walls of plants. Besides its structural role, cellulose can be hydrolysed by plant and microbial cellulases and in the case of pathogenic microorganisms the products of hydrolysis can be a source of signalling molecules to plant defence induction and of nutrients (Walton, 1994). In this study, taking endocellulase activity as an enzymatic model, the possibility is tested that variations in fungal colonization due to different plant capacities to form AM, can be a good experimental system to identify hydrolytic enzymes important to root colonization. To further elucidate the relationships between cellulase activity and fungal colonization we used pea mutants that have been identified in respect of their susceptibility to AMF colonization, and have been successfully used previously in physiological and biochemical studies of AM (Peterson and Guinel, 2000; Marsh and Schultze, 2001).

Because ethylene has a role in the modulation of the cell-wall modification process, such as fruit ripening and abscising flowers (Lashbrook et al., 1994; del Campillo and Bennett, 1996) together with the putative role of ethylene regulating both rhizobial and arbuscular mycorrhizal symbiosis (Guinel and Geil, 2002), it was hypothesized that ethylene could be a regulator of cellulase production and/or activity during AM development. We investigated here if the pea mutant line E107 which is altered in its symbiotic features and ethylene responses (Guinel and LaRue, 1992; Resendes et al., 2001) should exhibit alterations in the cellulase pattern of activity.

The present study shows data about the quantitative and qualitative endocellulase activity in roots of different pea mutants altered in their symbiotic abilities, finding a positive relationship between fungal colonization and endocellulase activity in roots.

## 2. Material and methods

### 2.1. Plant culture and inoculation procedures

Seeds of wild type pea (*Pisum sativum* vars. Sparkle and Finale) and symbiotic mutants E2 (*sym 5*) (Guinel and LaRue, 1991; Kneen and LaRue, 1988), E107 (*brz*) (Guinel and LaRue, 1992) and RisNod20 (sym *19*) (Engvild, 1987; Borisov et al., 2004) were surface-sterilized with $HgCl_2$ for 10 min, thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings were planted in the compartments described below and grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 $E\,m^{-2}\,s^{-1}$, 400–700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Plants were watered from below and fed with Long Ashton nutrient solution containing 25% of the P concentration (Hewitt, 1966).

The soil was steam-sterilized and mixed with sterilized sand, vermiculite and peat in equal amounts. The soil was a grey loam soil obtained from the grounds of the Estación Experimental del Zaidín (Granada, Spain). The soil had a pH of 8.1 in a 1:1 soil:water ratio. The P, N and K were determined using the methods of Mingorance (2002). $NaHCO_3$-extractable P was 6.2 $mg\,kg^{-1}$, N was 2.5 $mg\,kg^{-1}$and K was 132 $mg\,kg^{-1}$. The soil texture was 358 $g\,kg^{-1}$ sand, 436 $g\,kg^{-1}$ silt, 206 $g\,kg^{-1}$ clay and 18 $g\,kg^{-1}$ organic matter.

The assays of mycorrhization were performed in the three compartment system for hyphal-based inoculation described by Pinior et al. (1999) according to the original method developed by Wyss et al. (1991). The compartments were filled with the steam sterilized substrate described above. The central compartment contained the inoculum of *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 12). This source of inoculum consisted of one month old onion plants previously infected with soil, spores, mycelium and infected root fragments from an open pot culture of sorghum plant (*Sorghum vulgare* L.). The central compartment was separated from the lateral compartments by a nylon screen (60 μm mesh size) which allowed hyphae that come from onion infected roots, but not roots, to pass through. Each experiment began after joining the lateral compartments with the central compartment containing the inoculum. This experimental system led to a rapid and near synchronous infection of the root system of pea plants in the lateral compartments by hyphae from the central compartment. In control AM non-inoculated treatments, the onion plants growing in the central compartment were inoculated with a soil filtrate (Whatman No. 1 filter paper) obtained from the soil containing spores, mycelium and infected roots of sorghum plants. The filtrate contained common soil microorganisms, but no propagules of AM fungi.

Plants were harvested 20 days after inoculation, the root system was washed and rinsed several times with sterilized distilled water and cut into three. One part of the root was used to estimate the dry weight, the second part for enzymatic activity determinations and the third was cleared and stained for microscope examination (Phillips and Hayman, 1970). The percentage of total root length colonized by AM fungi was measured according the method of Giovannetti and Mosse (1980). The arbuscule colonization, and appressoria numbers in roots were measured under a microscope in 30 random pieces of 1 cm in length per stained root (Ocampo et al., 1980).

### 2.2. Ethylene quantification

Ethylene content in roots was measured by placing the excised root system in a 20 ml tube, closed with a rubber stopper, and incubated for 1 h at room temperature. The accumulation of ethylene in each tube was determined from three different samples of 1 ml taken with a syringe from the tube. The determination was carried out in a gas chromatograph (Hewlett Packard 5890) fitted with a flame ionization detector, using commercial ethylene as a standard for identification and quantification.

### 2.3. Preparation of extracts for enzyme assays

Roots (1 g fresh weight) were pulverized in a mortar under liquid nitrogen. The resulting powder was homogenized in 3 ml of buffer B (100 mM Tris-HCl buffer (pH 7) plus 0.02 g polyvinyl-polypyrrolidone (PVPP), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM $\beta$-mercaptoethanol, 0.15 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.3% (wt/vol) X-100 Triton. Sodium azide (0.03%) was added to all solutions. The liquid was filtered through several layers of cheesecloth and centrifuged at 20 000 *g* for 20 min. The supernatant fraction was designated fraction B and contains mostly soluble cellulase. The pellet was now homogenized in 3 ml of buffer B plus 1 M NaCl and centrifuged again to remove the residual cellulase bound to the cell-wall. This supernatant fraction was designated fraction C. Both fractions were dialyzed (Spectra/Por membrane, MWCO: 6–8000) against several hundred volumes of the same diluted extracting solutions (1:9, vol/vol) for 16 h at 4 °C. The samples were then frozen until used.

Total proteins were measured using a Bio-Rad kit with BSA as standard (Bradford, 1976).

### 2.4. Enzyme assays

The extracts were assayed to determine the endocellulase activity by the viscosity method (Rejón-Palomares et al., 1996) using cellulose as substrate. The reduction in viscosity was determined at 30 min intervals. Approximately 0.8 ml of the reaction mixture was sucked from a 2 ml tube into a 1-ml syringe through its needle and the time taken for the meniscus to move from the 0.70 to 0.20 ml mark (About 1–3 min) was recorded. The reaction mixture in the 2 ml tube contained 1 ml of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5) and 0.2 ml of enzymatic fraction. Viscosity reduction was determined at 37 °C. One unit of enzyme activity was expressed as specific activity (U/mg protein; U is the reciprocal of time in h for 50% viscosity loss $\times 10^3$) (Rejón-Palomares et al., 1996). Controls for all enzyme assays were autoclaved enzyme supernatants and autoclaved buffers.

### 2.5. Statistical analysis

The data were subjected to one-way ANOVA. The mean values of five replicate samples were compared using the Duncan's multiple range test ($P \leqslant 0.05$). Percentage data were subjected to arcsine transformation before analysis.

## 3. Results

The mycorrhizae characterization and quantification of different plant phenotypes was done according to the criteria of root length colonization and appressoria number per cm of root (Fig. 1). The wild type Sparkle and Finale were colonized in a normal manner. Compared with the parent Sparkle, the colonization of E2 and E107 genotypes resulted in a decrease in both percentage of root length colonization and arbuscules. Furthermore, the number of appressoria per cm in colonized roots was equal in E2 and E107, and significantly less than in Sparkle wild type. The percentage of colonization and arbuscules were higher in E2 genotype than in the E107. No significant difference in the appressoria number in root was found between the parent Finale and the RisNod20 mutant, but never hyphae were unable to penetrate the epidermis and colonize the cortex in this mutant.

The ethylene content in roots was measured to investigate a potential effect of the ethylene in endocellulase activity in the root of each mutant. No changes in ethylene content were observed between inoculated and non-inoculated roots in each plant genotype, but E107 mutant showed a higher content of ethylene than Sparkle and E2 lines (Fig. 2). These results suggest that the higher ethylene content in E107 mutant could be responsible for the lower mycorrhization level reached in this mutant.

The endocellulase activity in plant roots of peas inoculated with *G. mosseae* was higher than in non-inoculated controls in all of the plant genotypes assayed, except for the RisNod20 mutant (Fig. 3), where AM fungal penetration does not take place. The highest endocellulase activity of pea roots inoculated with *G. mosseae* or non-inoculated was found in the enzymatic fraction B, obtained without NaCl, but the clearest differences between AM inoculated and non-inoculated roots were observed in the enzymatic fraction C, obtained with high NaCl concentration. In all cases the endocellulase activity from fraction C of inoculated roots of parent lines was higher than activity in their mutants.

Clearly, the endocellulase activity of the enzymatic fractions C in Sparkle parent and their mutants (Fig. 3a) show the same pattern of behaviour than the percentage of colonization and arbuscules in these plants (Fig. 1a).

## 4. Discussion

The intracellular root colonization by AMF creates a new interface compartment composed of membranes from both partners separated by apoplastic material containing

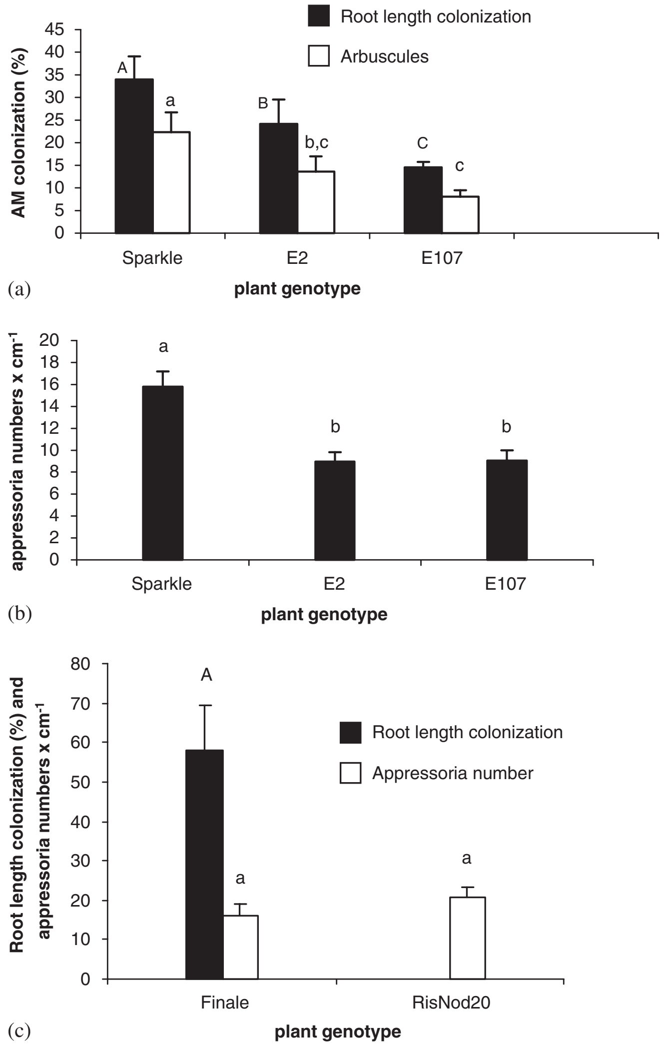

Fig. 1. AM colonization in different pea (*Pisum sativum*) genotype plants 20 days after inoculation with *Glomus mosseae*. Root length colonization and arbuscule occupancy (a) and appressoria numbers × cm$^{-1}$ (b) were analysed in Sparkle wild type and E2 and E107 mutants. Wild type Finale and RisNod20 mutant were compared according to root length colonization and appressoria numbers (c). The vertical bars are the SE of mean. Bars with the same letter are not significantly different according to Duncan's multiple range test ($P \leqslant 0.05$).

molecules common in the plant primary wall, such as cellulose, pectin, xyloglucan, and HRGP which are not assembled into a fully structured wall (Bonfante, 2001; Balestrini and Bonfante, 2005). The low lytic activity of plant and/or fungal enzymes on these molecules could alter the structure of plant cell-walls allowing fungal growth without weakening the cell-wall. Evidence of this is the low production of plant cell-wall-degrading enzymes by AMF (García-Romera et al., 1991; García-Garrido et al., 1992), and the activation by the plant of mycorrhiza-specific genes of plant cell-wall-degrading enzymes (Maldonado-Mendoza et al., 2005).

Endoglucanase and exoglucanase enzymes have been shown to increase in AM plants at the beginning of entry point formation and arbuscule development (García-Garrido et al., 1992). Also, activation of a cellulase gene, the *MtCel1*, has been associated specifically with cells that contain arbuscules in *Medicago truncatula* roots colonized

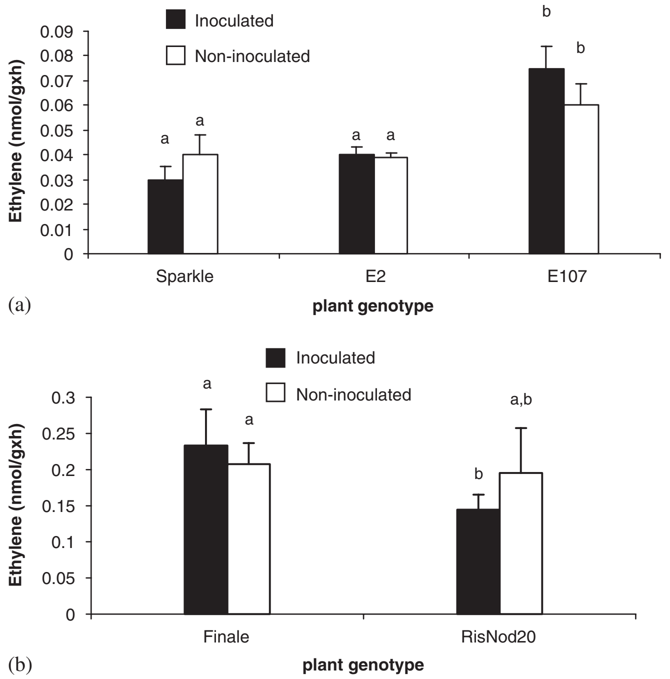

Fig. 2. Quantitative determination of ethylene in roots of Sparkle wild type and E2 E107 mutants (a) or Finale and RisNod20 genotype plants (b), non-inoculated or inoculated with *Glomus mosseae*. The vertical bars are the SE of mean. Bars with the same letter are not significantly different according to Duncan's multiple range test ($P \leqslant 0.05$).

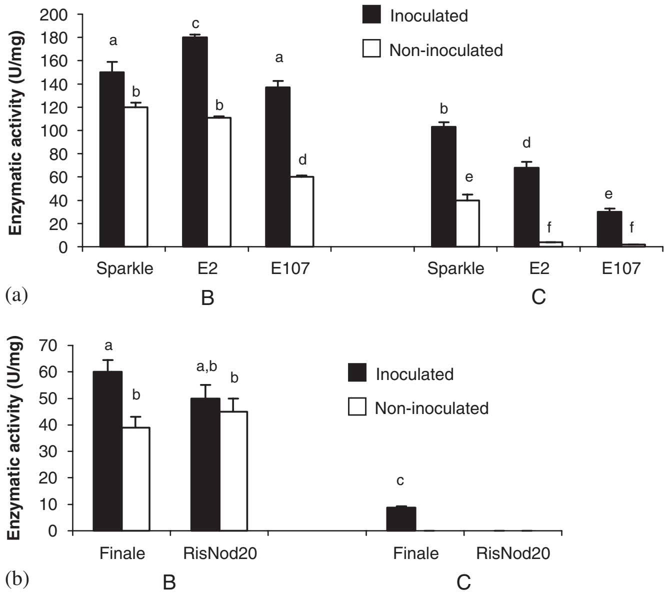

Fig. 3. Cellulase activity in soluble (B) and residual (C) protein fractions of roots of Sparkle wild type and E2, E107 mutants (a) or Finale and RisNod20 genotype plants (b), non-inoculated or inoculated with *Glomus mosseae*. The vertical bars are the SE of mean. Bars with the same letter are not significantly different according to Duncan's multiple range test ($P \leqslant 0.05$).

by *G. versiforme* (Liu et al., 2003). Therefore, it is logical to predict that cellulase activity is involved in cell-wall modifications during AM colonization and is induced in mycorrhizal roots and processes that alter mycorrhization could also alter the pattern of cellulase production in roots. Our results clearly corroborate this supposition, since the increase of activity is associated with fungal infection and their capacity of cortex colonization. RisNod20, a $\text{Myc}^{-1}$ mutant characterized by a normal pre-infective behaviour against AM fungi, but lacking an infective stage (Borisov et al., 2004) showed the same pattern of cellulase activity as non-inoculated plants.

The phenotypic description of a number of symbiotic mutants (mutated in SYM genes) supports genetic evidence that the penetration of the root epidermis and colonization of the cortex are two key events in the development of both rhizobia and AM fungi colonization (Guinel and Geil, 2002; Jacobi et al., 2003a, b). In the case of the AM symbiosis, relationships between morphological cell-type events and genetic loci mutations have been established, mainly in *Lotus japonicus* and *P. sativum* (Parniske, 2004). The penetration of the epidermis by the fungal hyphae requires the formation of a cleft between the walls of two adjacent epidermal cells. This process is regulated by the plant, perhaps by mediation of hydrolytic enzymes, and in the case of *L. japonicus* the participation of *LjSym15* is essential (Demchenko et al., 2004). After the penetration of the epidermis a successful fungal accommodation in the inner and outer cell layer of cortical cells is required. Cytological and genetic studies on *L. japonicus* showed that the *SYMRK* and *LjSym4* genes are essential for this process (Bonfante et al., 2000; Demchenko et al., 2004; Novero et al., 2002). Probably the *brz* (E107) gene of *P. sativum* has a similar function, because the LjSym4 phenotype is very similar to that of the brz mutant (Resendes et al., 2001).

In this context, the results obtained in our study corroborate previous studies of the low mycorrhization phenotype of E107 mutant (Resendes et al., 2001) and first demonstrate a low mycorrhization phenotype of E2 mutant, measured as intensity of colonization and appressoria formation per cm of roots. Furthermore, the results showed a positive correlation between cellulase activity in roots and mycorrhization, mainly in protein extracts obtained with high NaCl concentration. It is logical to speculate that the residual cellulase would be associated with the cell-wall and represent the enzymatic fraction of cellulase actively engaged in altering the cell-wall. This correlation is observed basically at quantitative level because no great changes in the pattern of cellulase bands in polyacrylamide gels were observed between colonized roots of the different phenotypes studied (data not shown). These results suggest that the increase in cellulase activity in mycorrhizal roots is associated mainly with epidermis penetration, hyphae spread within the cortex and arbuscule formation.

The cell-wall modifications during AM colonization may be similar to those occurring in other plant physiological events such as fruit ripening, and some genes induced in the AM symbiosis, that encode unknown proteins, are similar to genes induced during ripening (Liu et al., 2003). Phytohormone molecules, such as ethylene, act as regulators of hydrolytic activity in the process of cell-wall modification, such as fruit ripening and abscising flowers (Lashbrook et al., 1994; del Campillo and Bennett, 1996) and it should be possible that they could exercise the same function during AM formation. It is assumed that ethylene could play an important role in arbuscular mycorrhizal symbiosis (Ishii et al., 1996; Geil et al., 2001; Geil and Guinel, 2002). In this sense, the low mycorrhization phenotype of E107 plants could be due in part to high ethylene content in the root, as the data shown here about measures of ethylene content in roots demonstrate. We show in our result that ethylene production in plant roots was not altered by the mycorrhization. In this sense several studies show contradictory results since some of them showed that host ethylene production is decreased due to AM formation (Cruz et al., 2000; Besmer and Koide, 1999), presumably by ACC oxidase inhibition (McArthur and Knowles, 1992). In contrast to these results, other reports showed no effect (Vierheilig et al., 1994) or enhancements of the ethylene level in mycorrhizal roots (Dugassa et al., 1996). In our experiments the ethylene content in roots was measured punctually at the end of the experiment of mycorrhization and possible changes in the ethylene content in early stages are not determined. Furthermore, the decrease in ethylene sensitivity by blocking ethylene perception, but not ethylene level, is sufficient to produce the inhibition of the ethylene function (Guo and Ecker, 2004). Therefore, not only the ethylene level, but also ethylene sensibility determines ethylene action. In this context, Guinel and LaRue (1992) have shown that ethylene action inhibitors partly restore the nodulation phenotype of E107 mutant. Similarly, the E2 mutant has an ethylene-sensitive nodulation phenotype and exogenous ethylene inhibitors increased nodulation of this low-nodulating pea mutant (Fearn and LaRue, 1991).

Our results suggest that ethylene could participate in the restriction of mycorrhization in E107 plants but apparently does not affect the specific cellulase activity in roots during mycorrhiza formation. An inverse relationships between ethylene content and quantitative cellulose activity in roots can be detected, independently of the inoculation or not by the AM fungus, because the lowest activity among wild type Sparkle and its mutants was observed in mutant E107, having the highest level of ethylene production. At the same time, when comparing wild type plants Finale and Sparkle, Finale showed lower activity than Sparkle, and again Finale produce more ethylene than Sparkle. It seems possible that ethylene content could be important to the basal endocellulase activity in roots. When plants were challenged with the AM fungus the percentage of increase in activity in each type of plant was similar independently of their ethylene content. These finding suggests that no relationships was found between ethylene content and qualitative and/or quantitative increase in cellulase activity in mycorrhizal roots respect to non-mycorrhizal roots. It is interesting to note that phytohormone, including ethylene, could differentially regulate cellulase genes and enzyme activity (del Campillo and Bennett, 1996; Ferrarese et al., 1995; Lewis and Varner, 1970), depending of the specific cell-wall modification process and physiological conditions under it which takes place.

In conclusion, a positive relationship between the extent of epidermis penetration by the fungus, (hyphae spread through the cortex, and arbuscule formation) and cellulase activity in roots has been demonstrated. The increase in activity is dependent on the symbiotic phenotype features of the plant (penetration capacities, intensity of arbuscules, etc.).

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