

## PRODUCTION OF PECTOLYTIC ENZYMES IN LETTUCE ROOT COLONIZED BY *GLOMUS MOSSEAE*

I. GARCIA-ROMERA, J. M. GARCIA-GARRIDO, E. MARTINEZ-MOLINA and J. A. OCAMPO\*

Estacion Experimental del Zaidin, Prof. Albareda 1, Granada 18008, Spain

(Accepted 25 November 1990)

**Summary**—The type of extraction solution used influences pectolytic enzyme recovery from *Lactuca sativa* root colonized with *Glomus mosseae*. The most suitable extraction buffers are 250 mM NaCl for pectin esterase and 50 mM citrate-phosphate plus polyvinyl-pyrrolidone for polygalacturonase, endo-polygalacturonase, polymethylgalacturonase, endo-polymethylgalacturonase, pectin and pectate lyase. The inclusion of glycine and urea to citrate-phosphate buffer decreases polygalacturonase activity but enhances endo-polymethylgalacturonase activity. Mycorrhizal colonized roots possess more pectin esterase, with optimum production at pH 7 and endo-polymethylgalacturonase but not more polygalacturonase, polymethylgalacturonase endo-polygalacturonase pectin and pectate lyases, than non-colonized roots. This fact suggests that these enzymes may participate in the process of penetration and colonization of root by the vesicular-arbuscular mycorrhizal fungus *G. mosseae*.

### INTRODUCTION

Most phytopathogenic fungi and bacteria are known to produce enzymes that degrade pectic substances (Albersheim and Anderson-Prouty, 1975). Those enzymes that degrade the  $\alpha$ -1,4 linkages between galacturonosyl moieties in polymers of galacturonic acid play a fundamental role in pathogenesis (Garibaldi and Bateman, 1971). Research has shown that the degradation of pectin is due to the action of a complex of enzymes, including polygalacturonase, pectin esterase and pectin lyase (Collmer and Keen, 1986). The production of pectolytic enzymes has been observed not only in parasites but also in mutualistic microorganisms such as *Rhizobium*, and strongly support the importance of these cell-wall degrading enzymes produced by *Rhizobium* in the infection process of plant roots (Martinez-Molina and Olivares, 1982).

The biochemical mechanisms by which VA mycorrhizal fungi penetrate host tissues are still unknown, but electron microscope observations of fungal penetration into roots indicate that these fungi are probably able to degrade cell walls (Kinden and Brown, 1975; Bonfante-Fasolo, 1984). The presence of pectolytic enzymes in *Glomus mosseae* spores suggests the possible involvement of these enzymes in the colonization process (Garcia-Romera *et al.*, 1990). However, the ability of microorganisms to produce pectic enzymes *in vitro* constitutes no proof of their pathogenicity. Some microorganisms able to produce pectic enzymes on synthetic nutrient media did not always possess the ability to produce them *in vivo*. Moreover, attempts to demonstrate pectinase production in extracts from VA mycorrhizal tissue have not been successful (Anderson, 1988). Confirmation of the production of pectolytic enzymes may be impeded by the very low levels of enzyme production,

as found for other mutualistic microorganisms (Angle, 1986). Other causes may lie in the chemical composition of the cell walls of plants which repress or control the production of pectic enzymes (Rexova-Benkova and Markovic, 1976). Besides this protective mechanism, there is the possibility of the effect of other inhibitors, including phenolic compounds (Mullen and Bateman, 1971; Dazzo and Hubbell, 1974), which may be produced during the colonization process or during the extraction of enzymes from colonized tissues. The type of extraction solution is known to influence enzyme recovery (Morales *et al.*, 1984).

Since the effect of VA colonization on enzyme production is currently unknown, the following study was initiated, however, to study this problem methodologies in enzyme extraction first had to be developed. The objective of this study was to determine whether pectolytic enzymes are produced in lettuce plants colonized with *G. mosseae*.

### MATERIAL AND METHODS

Plants were grown in 300 ml capacity open pots of soil collected from Granada Province, Spain. The soil was a reddish-brown type, pH 7.6 (see Garcia-Romera and Ocampo, 1988), and was steam-sterilized and mixed with sterilized sand in a 1:1 (v/v) proportion.

Lettuce (*Lactuca sativa* cv. Romana) was used as the test plant. Seeds were sown in moistened sand. After 2 weeks, seedlings were transplanted to the pots and grown under greenhouse conditions (natural light was supplemented by Sylvania incandescent and cool-white lamps, 400 nmol m<sup>-2</sup> s<sup>-1</sup>, 400–700 nm; with a 16–8 h light–dark cycle at 25–19°C and 50% r.h.). Plants were watered from below using a capillary system, and fed with a phosphate-free nutrient solution (Hewitt, 1952).

\*Author for correspondence.

The VA inoculum consisted of 5 g of rhizosphere soil from either maize (*Zea mays* cv. Calderon) or alfalfa (*Medicago sativa* cv. Aragon) plant pot cultures of a isolate of *G. mosseae* which contained spores, mycelium and colonized root fragments. Uninoculated plants were given filtered leachings from the inoculum soil. Soil filtrate (Whatman No. 1 filter paper) from the rhizosphere of mycorrhizal plants was added to VA uninoculated treatment. The filtrate contained common soil microorganisms, but no propagules of *G. mosseae*. All assays were repeated 3 times (twice using *G. mosseae* inoculum from maize and using alfalfa inoculum, 20–25 spores  $g^{-1}$  soil, in the remaining replication) with similar results.

Plants were harvested after 9 weeks. The root system was washed and rinsed 3 times with sterilized distilled water and parts of the root system from each of the five replicate group of pots were cleared and stained (Phillips and Hayman, 1970), and examined under a compound microscope. The percentage of total root length which was colonized with VA mycorrhiza was measured (Ocampo *et al.*, 1980). The rest of the roots (20 g) were finely pulverized in a mortar under liquid nitrogen. The resulting powder was homogenized in 40 ml of the following solutions: 250 mM NaCl (Hancock, 1966); 50 mM citrate-phosphate (CP) buffer (pH 7) (Martinez-Molina and Olivares, 1982); 50 mM CP buffer plus 13 g of polyvinyl pyrrolidone (PVPP) (Sigma); 50 mM buffer CP (pH 7) plus 1% glycine and 1 M urea (Morales *et al.*, 1984); 100 mM Tris-HCl buffer (Garcia-Romera *et al.*, 1990). Sodium azide (0.03%) was added to all solutions to prevent microbial growth. The liquid was filtered through several layers of cheesecloth. The extract was centrifuged at 20,000  $g$  for 15 min, and the pellet resuspended and washed by centrifugation with the same buffer three times. The supernatant was treated with ammonium sulphate up to 80% of full saturation (to precipitate pectinase protein). The solution was kept for 5 h at 4°C and centrifuged once more as described above. The supernatant was discarded. The sediment was dissolved in a small volume of the same extractant solution and dialyzed against 2 l of the same extractant solutions for 16 h at 4°C. The samples were then frozen until use.

The extracts were assayed to determine the activities of pectin esterase (PE) (EC 3.1.1.11), endopolygalacturonase (endo-PG) (EC 3.2.1.15), exopolygalacturonase (PG) (EC 3.2.1.67), pectate lyase (PAL) (EC 4.2.2.9) and pectin lyase (PL) (EC 4.2.2.10). The activities of endo-PG and PG on pectin were designated as endo-polymethylgalacturonase (endo-PMG) and polymethylgalacturonase (PMG), respectively.

#### Enzyme assays

The substrates used in the analyses of pectic enzymes were Na polygalacturonate and citrus pectin (Sigma). Three general methods were employed for the detection of pectic substance depolymerases: reducing groups released, viscosity reduction and increase in absorption at 235 nm. Reducing groups were measured by the procedures of Nelson (1944) and Somogyi (1952). The increase in reducing groups was determined in reaction mixtures containing

0.3 ml 1% (w/v) pectin or sodium polygalacturonate, 0.3 ml 50 mM potassium phosphate, pH 6.0, 2 mM EDTA (to inhibit lyases), and 0.4 ml enzyme. One unit of enzyme was defined as the amount of enzyme which released 1.0  $\mu$ mol of galacturonic acid  $h^{-1}$  at 37°C. Viscosity reduction was determined in a Cannon-Fenske viscosimeter (5354/4) at 37°C. The reaction mixture (6 ml) contained 1% substrate enzyme, 50 mM CP buffer (pH 5) and 1 ml enzyme. One unit of enzyme activity was expressed as relative activity (RA: reciprocal of time in h for 50% viscosity loss  $\times 10^3$ ) (Bateman, 1963). The activities of PL and PAL were measured by recording the increase in absorption at 235 nm of a pectin or sodium polygalacturonate solution, respectively (Starr and Moran, 1962). The reaction mixture contained 1 ml 0.5% solution of substrate; 0.5 ml 50 mM Tris-HCl buffer (pH 8.5), supplemented with 1 mM  $CaCl_2$ ; and 1 ml enzyme preparation. The reactions were stopped by adding 0.2 ml 9%  $ZnSO_4 \cdot 7H_2O$ , mixing well, and centrifuging at 10,000  $g$  for 10 min. One enzyme unit was defined as a change of 0.01 absorbance units in 1 h.

Pectin esterase (PE) was assayed by measuring the release of acid groups from pectin. The reaction mixture consisted of 15 ml of 1% pectin (pH 5 and pH 7) and 2.5 ml enzyme solution dialysed against 250 mM NaCl or water to remove buffers. After reaction times of 10, 20 and 40 min at 37°C, the enzyme-substrate mixture was titrated with 0.02 *N* NaOH to the pH recorded at zero time. However, the 1% pectin substrate solution was made up in 250 mM NaCl and in water so that enzymatic activity could be determined in both the presence and absence of salt. One unit of PE activity was defined as the amount of enzyme that required the addition of 1 microequivalent of NaOH  $h^{-1}$  to maintain the reaction mixture at pH 5 or at pH 7. Controls for all enzyme assays were autoclaved enzyme extracts and buffers, and 0.03% of sodium azide was added to all reaction mixtures. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as the standard.

#### RESULTS

After 9 weeks of growth VA inoculated and uninoculated plants were healthy and no disease symptoms were observed. Microscope observations of stained roots showed no presence of fungi in uninoculated control and only VA mycorrhizal structures in VA inoculated plants. Lettuce plants inoculated with *G. mosseae* reached  $42 \pm 7\%$  of VA root length colonization.

Root proteins extracted in 250 mM NaCl showed more PE activity than those prepared with Tris-ClH or CP buffers (Table 1). The extracts prepared with CP buffer demonstrated more enzymatic activity than those prepared with Tris-ClH buffer. The inclusion of PVPP or glycine and urea in the CP buffer did not greatly affect the amount of activity obtained in their absence. When root extracts were prepared in 250 mM NaCl, mycorrhizal colonized roots contained more PE activity than non-mycorrhizal roots either in absence (data not presented) or in presence of NaCl (Table 1) in their reaction mixtures. When salt was

Table 1. Pectin esterase activity and the pH values in root of mycorrhizal and non-mycorrhizal lettuce plants extracted with different buffers

Buffer composition	Specific activities (units mg <sup>-1</sup> protein)			
	pH 5.5		pH 7	
	-M	+M	-M	+M
250 mM NaCl	9.3c	18.6d	27.4e	48.2f
50 mM CP (pH 7)	4.2b	5.4b	13.8d	12.6d
50 mM CP (pH 7) plus PVPP	4.1b	4.8b	12.6d	14.4d
50 mM CP (pH 7) plus 1% glycine and 1 M urea	5.4b	6.6b	19.2d	18.1d
100 mM Tris-CIH	1.1a	1.3a	8.4c	9.6c

-M = Minus mycorrhiza; +M = Plant inoculated with mycorrhiza. Values sharing the same letter are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

omitted from reaction mixtures, small amounts of activity were detected in extracts from colonized roots. When PE activity was measured as a function of the pH of the reaction mixture two peaks of activity appeared at pH 5 and at pH 7, with optimum PE production at pH 7 (Fig. 1).

Endo-PG and PG activities were detected in inoculated and non-inoculated plants, with activities in roots extracted with CP buffer higher than in roots extracted with 250 mM NaCl or Tris-CIH buffer. The addition of PVPP to the CP buffer enhanced endo-PG activity. However, the application of glycine and urea to CP buffer decreased PG activity. Endo-PG and PG activities were similar in mycorrhizal and non-mycorrhizal root extracts regardless of the extraction solution used (Table 2).

Endo-PMG activity (Table 3) in mycorrhizal roots extracted in CP buffer was higher than in roots extracted with 250 mM NaCl or Tris-CIH buffer, and more endo PMG was found in mycorrhizal than in non-mycorrhizal root extracts. The addition of PVPP to the CP buffer enhanced the activity in mycorrhizal plants. Although the inclusion of glycine and urea enhanced the enzymatic activity of plant roots, these

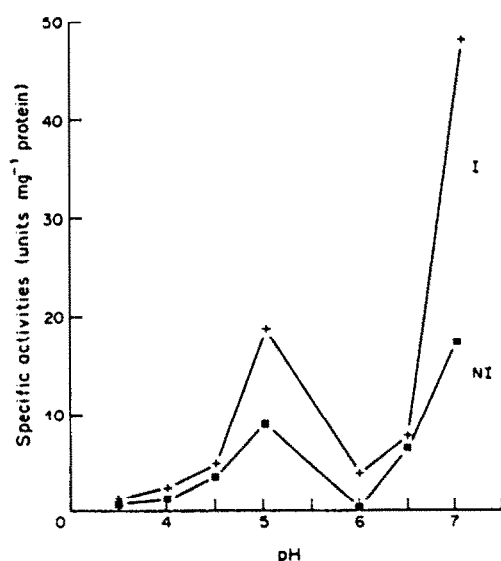


Fig. 1. Effect of pH on pectinesterase from mycorrhizal and non-mycorrhizal lettuce roots. I = Plant inoculated with mycorrhiza; NI = Noninoculated plant.

Table 2. Endo-PG and PG activities in root of mycorrhizal and non-mycorrhizal lettuce plants extracted with different buffers

Buffer composition	Specific activities (units mg <sup>-1</sup> protein)			
	endo-PG		PG	
	-M	+M	-M	+M
250 mM NaCl	2.0a	2.8a	0.4a, b	0.5a, b
50 mM CP (pH 7)	6.2b	7.8b	2.2c	2.1c
50 mM CP (pH 7) plus PVPP	10.8c	9.6c	2.4c	2.9c
50 mM CP (pH 7) plus 1% glycine and 1 M urea	6.4b	7.3b	1.0b	0.9a, b
100 mM Tris-CIH	1.7a	2.1a	0.1a	0.1a

Within endo-PG and within PG values sharing the same letter are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

substances did not influence the activity of extracts from mycorrhizal over non-mycorrhizal roots. We found that extracts prepared with CP buffer contained higher amounts of PMG (Table 3), PL and PAL proteins (Table 4) compared to those prepared with 250 mM NaCl or Tris-CIH buffer, however, the differences in these enzymatic activities between VA colonized and noncolonized roots were non-significant.

#### DISCUSSION

The production of hydrolytic enzymes by *G. mosseae* spores (Garcia-Romera *et al.*, 1990), suggested the possible involvement of these enzymes in the process of colonization as suggested in electron microscopic studies (Kinden and Brown, 1975; Bonfante-Fasolo, 1984; Jeanmougin *et al.*, 1987; Bonfante-Fasolo and Vian, 1989).

Table 3. Endo-PMG and PMG activities in root of mycorrhizal and non-mycorrhizal lettuce plants extracted with different buffers

Buffer composition	Specific activities (units mg <sup>-1</sup> protein)			
	endo-PMG		PMG	
	-M	+M	-M	+M
250 mM NaCl	4.3a, b, c	5.3b, c	1.2a	0.6a
50 mM CP (pH 7)	7.3c, d	27.3f	3.6b	3.1b
50 mM CP (pH 7) plus PVPP	10.2d	45.6g	2.4b	3.2b
50 mM CP (pH 7) plus 1% glycine and 1 M urea	15.4e	18.6e	6.6c	5.4c
100 mM Tris-CIH	1.7a	9.6d	0.6a	0.6a

Within endo-PMG and within PMG values sharing the same letter are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

Table 4. PL and PAL lyase activities in root of mycorrhizal and non-mycorrhizal lettuce plants extracted with different buffers

Buffer composition	Specific activities (units mg <sup>-1</sup> protein)			
	PL		PAL	
	-M	+M	-M	+M
250 mM NaCl	8.6b	7.3b	11.4a	14.2a
50 mM CP (pH 7)	19.4c	19.2c	59.1c	59.3c
50 mM CP (pH 7) plus PVPP	17.6c	20.2c	81.2d	72.7d
50 mM CP (pH 7) plus 1% glycine and 1 M urea	24.6d	27.3d	48.8c	51.1c
100 mM Tris-CIH	3.2a	2.7a	8.6a	10.2a

Within PL and within PAL values sharing the same letter are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

Considerable attention has been devoted to the study of the role of pectolytic enzymes in physiological and pathological changes of plants, but research of these enzymes in plant roots are scarce (Rexova-Benkova and Markovic, 1976), only data from soybean and carrot roots are available (Verma and Zogbi, 1978; Konno *et al.*, 1989). Endo-PG activity from crude extracts of soybean roots prepared with 250 mM phosphate buffer (0.05 units  $\text{mg}^{-1}$  root) and exo-PG activity from culture filtrate of carrot cell culture (0.46 specific units  $\text{mg}^{-1}$  protein) were similar to endo-PG activity obtained in crude extracts with Tris-HCl buffer from lettuce roots (data not presented) or to exo-PG activity in lettuce roots extracted with 250 mM NaCl or Tris-HCl buffer (Table 2).

The type of extraction solution used influences enzyme recovery (Morales *et al.*, 1984). From the results obtained, the most suitable extraction buffers was 250 mM NaCl for PE and CP for the other pectolytic enzymes. A substantial proportion of PE is bound to the cell wall and is released on treatment with salt solution (Hancock, 1966). The different enzyme extractabilities of the solutions used in our experiments could be due to the different localization or properties of binding to cell wall. Some pectolytic enzymes produced in colonized tissue have been reported to be inactivated by phenol oxidation during extraction (Dazzo and Hubbell, 1974). The successful extraction of some pectolytic enzymes with CP plus PVPP from roots may be due to the insoluble complexes which PVPP forms with phenolic compounds by hydrogen bonding. The positive effect of glycine and urea on endo-PMG and PMG activities may be due to the effect of these compounds in dissolve the precipitate of enzymes after the ammonium sulfate treatment (Morales *et al.*, 1984). However, the negative effect on PG activity could be due to the usual negative effect of urea on enzyme activity (Hippel and Wong, 1964), or to the inactivation of some polygalacturonases by glycine (Kertesz, 1951).

Mycorrhizal colonized roots possess more PE than non-colonized roots with optimum at pH 7 which could indicate that this activity is of plant origin. However, the original view that pectinesterases from higher plants differ from those of microbial origin in optimum pH (in the acid range for fungal pectinesterases) is of limited validity. The optimum pH for all pectinesterases of higher plants is pH 7, but for some of the microbial pectinesterases, pH optima in the alkaline region have also been reported (Rexova-Benkova and Markovic, 1976).

The fact that mycorrhizal colonized roots possess more PE and endo-PMG, but not more PMG, endo-PG, PG, PL and PAL, than non-colonized roots, with the extractant solutions used in this experiment, may mean that PE and endo-PMG are the main pectolytic enzymes involved in the colonization of the root by the fungus. However, spores and external mycelium of *G. mosseae* contain PMG, endo-PG, PG, PL and PAL enzymes (unpublished results). Thus, the absence of differences between these degradative enzymes in root extracts of VA inoculated and uninoculated plants is not conclusive evidence for their non-participation in the colonization process. In fact, the possible production of these enzymes may depend

on the stage of root colonization by the fungus, as shown for other enzymes (Spanu and Bonfante-Fasolo, 1988). Although we found that CP buffer plus PVPP was the best extractant solution, the amount of PMG, endo-PG, PG, PL and PAL activities produced by the fungus may be too low to be detectable at this stage of mycorrhizal colonization with the extraction procedures used in this study.

Our results do not allow us to unequivocally claim a role for pectolytic enzymes in the process of penetration and colonization of the root by the VA fungus. However, the fact that higher amounts of PE and endo-PMG activities were detected in colonized plants than in non-colonized plants strongly suggest that these enzymes may participate in the unknown process of colonization of plant root by VA mycorrhizal fungi.

*Acknowledgements*—Financial support for this study was provided by the Comision Interministerial de Ciencia y Tecnologia.

#### REFERENCES

- Albersheim P. and Anderson-Prouty A. J. (1975) Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. *Annual Review of Plant Physiology* **26**, 31–52.
- Anderson A. J. (1988) Mycorrhizae-host specificity and recognition. *Phytopathology* **78**, 375–378.
- Angle J. S. (1986) Pectic and proteolytic enzymes produced by fast- and slow-growing soybean rhizobia. *Soil Biology & Biochemistry* **18**, 115–116.
- Bateman D. F. (1963) Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extract of *Rhizoctonia*-infected tissues of bean. *Phytopathology* **53**, 197–204.
- Bonfante-Fasolo P. (1984) Anatomy and morphology of VA mycorrhizae. In *VA Mycorrhizas* (C. LL. Powell and D. J. Bagyaraj, Eds), pp. 5–33. CRC Press, Boca-Raton.
- Bonfante-Fasolo P. and Vian B. (1989) Cell wall architecture in mycorrhizal roots of *Allium porrum* L. *Annales des Sciences Naturelles, Botanique, Paris* **10**, 97–109.
- Collmer A. and Keen N. T. (1986) The role of pectin enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**, 383–409.
- Dazzo F. and Hubbell D. (1974) A quantitative assay of insoluble polyvinylpyrrolidone. *Plant and Soil* **40**, 435–439.
- Garcia-Romera I. and Ocampo J. A. (1988) Effect of the herbicide MCPA on VA mycorrhizal infection and growth of *Pisum sativum*. *Zeitschrift fur Pflanzenernahrung und Bodenkunde* **151**, 225–228.
- Garcia-Romera I., Garcia-Garrido J. M., Martinez-Molina E. and Ocampo J. A. (1990) Possible influence of hydrolytic enzymes on vesicular arbuscular mycorrhizal infection of alfalfa. *Soil Biology & Biochemistry* **22**, 148–152.
- Garibaldi A. and Bateman D. F. (1971) Pectic enzymes produced by *Erwinia chrysanthemi* and their effect on plant tissue. *Physiological Plant Pathology* **1**, 25–40.
- Hancock J. G. (1966) Degradation of pectic substances associated with pathogenesis by *Sclerotinia sclerotiorum* in sunflower and tomato stems. *Phytopathology* **56**, 975–979.
- Hewitt E. J. (1952) Sand water culture methods used in the study of plant nutrition. *Commonwealth Agricultural Bureau, Technical Communication No. 22*.
- Hippel P. H. and Wong K. Y. (1964) Neutral salts: the generality of their effects on the stability of macromolar conformations. *Science* **165** 577–580.
- Jeanmougin J., Gianinazzi-Pearson V. and Gianinazzi S. (1987) Endomycorrhizas in the *Gentiniaceae* I. Ultrastruc-

- tural aspects of symbiont and relationships in *Gentiana lutea*. *Symbiosis* **3**, 269–286.
- Kertesz Z. I. (1951) *The Pectic Substances*. Interscience, London.
- Kinden D. A. and Brown M. F. (1975) Electron microscopy of vesicular-arbuscular mycorrhizas of yellow poplar II. Intracellular hyphae and vesicles. *Canadian Journal of Microbiology* **21**, 1768–1780.
- Konno H., Yamasaki Y. and Katoh K. (1989) Extracellular exo-polygalacturonase secreted from carrot cell cultures. Its purification and involvement in pectic polymer degradation. *Physiologia Plantarum* **76**, 514–520.
- Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- Martinez-Molina E. and Olivares J. (1982) A note on evidence for involvement of pectolytic enzymes in the infection process of *Medicago sativa* by *Rhizobium meliloti*. *Journal of Applied Bacteriology* **52**, 453–455.
- Morales V. M., Martinez-Molina E. and Hubbell D. H. (1984) Cellulase production by *Rhizobium*. *Plant and Soil* **80**, 407–415.
- Mullen J. M. and Bateman D. F. (1971) Production of an endo-polygalacturonate trans-eliminase by a potato dry-rot pathogen, *Fusarium roseum* "Avenaceum", in culture and in diseased tissue. *Physiological Plant Pathology* **1**, 363–373.
- Nelson N. (1944) A photometric adaptation of the Somogyi method to the determination of glucose. *Journal of Biological Chemistry* **153**, 373–380.
- Ocampo J. A., Martin J. and Hayman D. S. (1980) Influence of plant interactions on vesicular-arbuscular mycorrhizal infection I. Host and non-host plant grown together. *The New Phytologist* **84**, 27–35.
- Phillips J. M. and Hayman D. S. (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**, 158–161.
- Rexova-Benkova L. and Markovic O. (1976) Pectic enzymes. *Advances in Carbohydrate Chemistry and Biochemistry* **33**, 323–385.
- Somogyi M. (1952) Notes on sugar determination. *Journal of Biological Chemistry* **159**, 19–23.
- Spanu P. and Bonfante-Fasolo P. (1988) Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum*. *The New Phytologist* **109**, 119–124.
- Starr M. P. and Moran F. (1962) Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. *Science* **135**, 920–921.
- Verma D. P. S. and Zogbi V. (1978) A cooperative action of plant and *Rhizobium* to dissolve the host cell curing development of root symbiosis. *Plant Science Letters* **13**, 137–142.