Biol Fertil Soils (1994) 17:51–56

ORIGINAL PAPER

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Phosphatase activity and cytokinin content in cowpeas (*Vigna unguiculata*) inoculated with a vesicular-arbuscular mycorrhizal fungus

Received: 4 January 1993

Abstract We examined the effect of a vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus pallidum Hall* on the phosphatase activity and cytokinin concentration in cowpea [*Vigna unguiculata* (L.) Walp] roots at successive stages of plant growth. Both acid and alkaline phosphatase activity were significantly ($P = 0.05$) higher in mycorrhizal than in non-mycorrhizal roots 30 days after inoculation. Similarly, the cytokinin content was significantly increased in mycorrhizal roots compared to non-mycorrhizal roots. Our study suggests that these biochemical changes may improve the growth of mycorrhizal cowpea plants.

Key words Vesicular-arbuscular mycorrhizae · *Glomus pallidum* · Cowpea · *Vigna unguiculata* · Cytokinin · Roots

Introduction

The beneficial effects of VAM fungi on plant growth have often been related to the uptake of immobile nutrients, especially P (Kucey et al. 1989; Bolan 1991). Mycorrhizal fungi improve P availability by solubilizing inorganic forms of P and by the mineralization of organic P (Hetrick 1989). The solubilization of P may be achieved by the release of organic acids and phosphatase enzymes (Mitchell and Read 1981; Harley 1989). In most cases increased plant growth in mycorrhizal plants has been correlated with increased P levels in the shoots (Mosse et al. 1976; Waidyanatha et al. 1979). Although increased P uptake may be a major part of nutrient response promoted by mycorrhizal associations, a mycorrhizal plant may use P nutrients more efficiently than a non-mycorrhizal plant (Bowen 1973).

Increased levels of phosphatases in mycorrhizal onion roots (Gianinazzi-Pearson and Gianinazzi 1976, 1978) and increased levels of cytokinin activity in mycorrhizal *Bouteloua gracilis* roots have been reported (Allen et al. 1980). Barea and Azcon-Aguilar (1982) have shown that *Glomus mosseae* produces plant growth-regulating substances in axenic cultures. Although limited studies have been carried out on the induction of VAM-specific phosphatases and plant growth regulator-like substances in a few crops, little information is available on the effect of VAM inoculation on phosphatase activity and on the production of plant growth regulators in legumes in general and the cowpea (*Vigna unguiculata*) in particular. Earlier, we reported increased growth responses and high yields due to VAM inoculation of cowpeas in both greenhouse and field studies. In these studies mycorrhizal cowpea plants produced higher shoot weights and shoot P levels than non-mycorrhizal plants (Ames et al. 1991; Thiagarajan et al. 1992). In the present study we examined the effect of the VAM fungus *Glomus pallidum* on phosphatase activities and cytokinin production in mycorrhizal cowpea roots at different stages of plant growth.

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Materials and methods

Plant growth conditions

For the enzyme studies the cowpea plants were grown in a clay loam soil (pH 7.0) with an available P concentration of 14.1 $\mu g\, g^{-1}$ and available N of 1.8 $\mu g\, g^{-1}$. The soil was autoclaved for 1 h for 2 consecutive days according to Aarons and Ahmad (1987) and placed in growth pouches (15×20 cm). Two surface-sterilized (Ahmad et al. 1981) cowpea (cv. Laura B) seeds were sown in each pot and the two plants were thinned to one after 1 week. The VAM fungus (*Glomus pallidum*) used was an effective strain isolated in Jamaica (Ames et al. 1991). The VAM fungal inoculum was prepared and cowpea seeds were inoculated as described previously (Ames et al. 1991). Control seeds were treated with the same amount of heat-killed inoculum. The plants were grown in a greenhouse and watered as necessary. Five replicates of each treatment were harvested 10, 20, 30, and 40 days after planting. The plants were uprooted and washed carefully in ice-cold water and the roots were used immediately for enzyme extractions. Samples of the roots

were cut at random and checked for mycorrhizal colonization according to Ames et al. (1991). The shoot material was oven-dried (70 °C) and weighed, then ground, digested, and the P content determined according to the method described previously (Ames et al. 1991).

For the cytokinin assay the cowpea plants were grown in sterile vermiculite supplemented with Ruakura nutrient solution (Smith et al. 1983) in a controlled environment chamber (16 h light at 30 °C, 8 h dark at 22 °C). *Glomus pallidum* spores were surface-sterilized for 2 min in 0.5% sodium hypochlorite and germinated on water agar. Cowpea seedlings were inoculated with five germinating spores of VAM fungus and carefully transplanted to growth pouches (15×20 cm) containing a sterile vermiculite and nutrient solution. The plants were grown in a growth chamber for 45 days. They were harvested at 15-day intervals and the roots were randomly checked for mycorrhizal colonization (Ames et al. 1991).

Enzyme assay

Phosphatase enzymes were extracted according to a modification of the method described by Barrett-Lennard and Greenway (1982). Root tissue (1 g) was homogenized in 5 ml 0.1 *M* sodium acetate buffer (pH 6.5) and centrifuged at 10000 *g* for 15 min. The supernatant was further subjected to ammonium sulphate precipitation (80%) and the pellet was redissolved in 1 ml acetate buffer. The semipurified enzyme was passed through a G_{75} Sephadex column (30 cm×1.5 cm; Pharmacia Fine Chemicals, Sweden) and eluted with 0.1 *M* acetate buffer (pH 6.5) at a speed of 1 ml min^{-1}. Fractions (3 ml) were collected and the protein was read at 280 nm and assayed for phosphatase activity. All operations were carried out at 4 °C in a cold room.

Phosphatase activity was assayed according to Dodd et al. (1987). The reaction mixture for alkaline phosphatase consisted of 0.2 ml enzyme extract + 0.4 ml 15 m*M* *p*-nitrophenol phosphate (pNPP) in 0.25 *M* TRIS-HCl buffer (pH 9.6), made up to 1 ml with the same buffer. For acid phosphatase the reaction mixture was the same except that the substrate was dissolved in 0.25 *M* acetate buffer (pH 5.7). The mixture was incubated at 37 °C for 30 min and the reaction was terminated by adding 1 ml 1 *M* NaOH. The absorbance was read at 410 nm in a spectrophotometer. Specific phosphatase activity was expressed as nmol nitrophenol released per mg protein per min. The protein was determined according to Lowry et al. (1951).

Extraction and assay of cytokinin

After thorough washing in sterile distilled water the root samples (5 g root material from mycorrhizal or non-mycorrhizal plants) was ground in 0.1 *N* NaOH (pH 10) and left for 1 h. The mixture was then extracted twice with ethyl ether. The solvent was evaporated in a rotary evaporator in a vacuum at 30 °C and the residue was dissolved in 1 ml absolute methanol. A known weight of authentic cytokinin (Kinetin, Sigma Chemicals) was also subjected to the same extraction procedure.

Twenty microlitres of extract were run through a C_{18} analytical column of high-performance liquid chromatography (HPLC Beckman System Gold with solvent module 126 and detector module 166) by using methanol: water (60:40) as eluents. The eluents were double-distilled and degassed at 60 °C in a vacuum. The flow rate was 1 ml min^{-1} and the cytokinins were detected at an ultraviolet wavelength of 254 nm (Challice 1974). For a callus tissue assay the callus was obtained from soybean epicotyl and maintained on Miller's medium (1965). A medium without cytokinin was supplemented with 100 µl cytokinin extract in methanol 100 ml^{-1} medium. Five pieces of 10 mg callus tissue in five replicates were kept in 100-ml flasks containing 50 ml medium. The flasks were kept in the dark at 25 °C for 25 days, and then the callus pieces were reweighed and the increase in callus weight calculated (Allen et al. 1980).

Results and discussion

Figure 1 a and b shows the sephadex column chromatography of acid and alkaline phosphatases from 40-day-old non-mycorrhizal and mycorrhizal roots, respectively. The column chromatography revealed the appearance of an extra peak that eluted at the 23rd fraction in mycorrhizal root samples which was absent in non-mycorrhizal root samples. In the early stages of plant growth (10th and

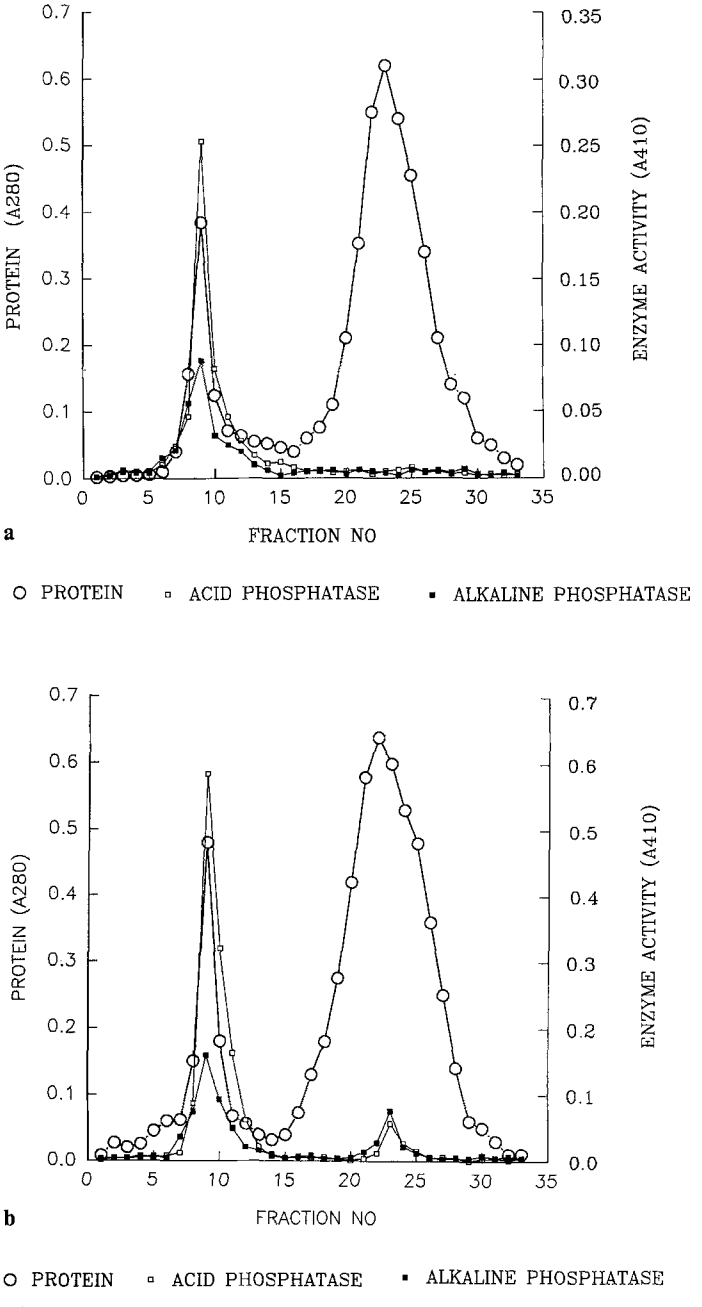
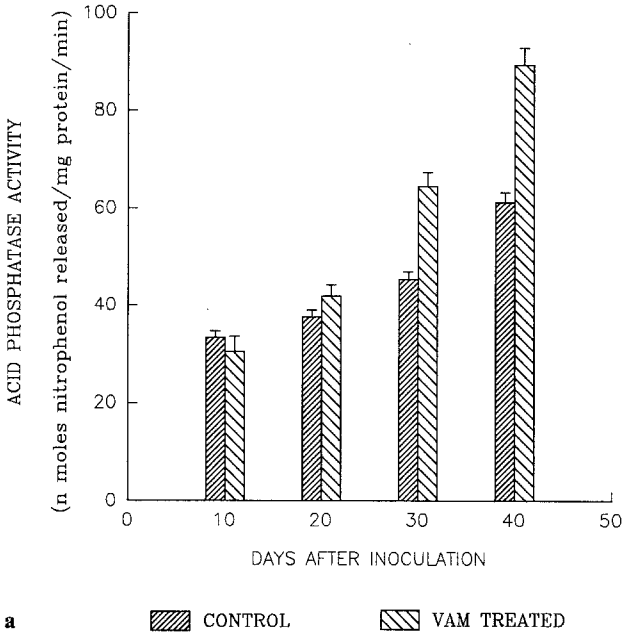


Fig. 1 Gel filtration chromatography of alkaline and acid phosphatase from non-mycorrhizal (**a**) and mycorrhizal (**b**) cowpea roots (40 days old). Elution was carried out on a Sephadex G_{75} column (1.5×30 cm) by using 0.1 *M* acetate buffer (pH 6.5) with a flow rate of 1 ml min^{-1}, and 3-ml fractions were collected

20th days) the elution pattern of acid and alkaline phosphatases in mycorrhizal root samples was similar to that of non-mycorrhizal samples (data not shown). When specific enzyme activities were assayed at various stages of plant growth, both acid and alkaline phosphatase activities were significantly increased in mycorrhizal roots compared with non-mycorrhizal roots on the 30th and 40th days (Fig. 2a, b). Increased phosphatase activities in mycorrhizal roots on the 30th and 40th days were positively correlated with the shoot P content ($r = 0.859$) and shoot dry weight ($r = 0.745$) (Table 1). This is further evidence that VAM fungi increase the shoot P content and shoot dry weight of mycorrhizal plants.



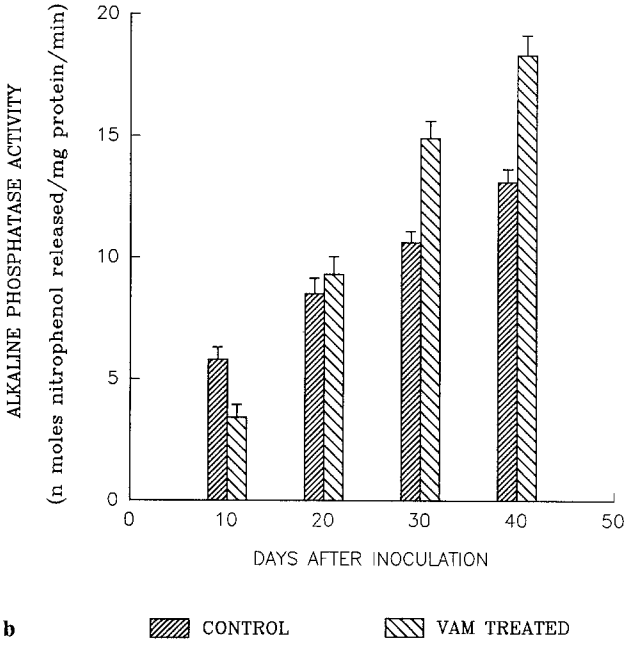


Fig. 2 Acid (**a**) and alkaline (**b**) phosphatase activity in mycorrhizal and non-mycorrhizal cowpea roots at successive stages of plant growth. *LSD*, Least significant difference; VAM, vesicular-arbuscular mycorrhizae

There are different views regarding acid and alkaline phosphatase activities in mycorrhizal plants. Gianinazzi-Pearson and Gianinazzi (1976) and Krishna et al. (1983) reported no significant increase in acid phosphatase activity in mycorrhizal roots. But in the same study significant increases in alkaline phosphatase activity were found in mycorrhizal plants. In contrast, Dodd et al. (1987) found a significant increase in acid phosphatase activity in mycorrhizal plants, the activity being dependent on the species of VAM fungus used for inoculation. In the present study increased acid and alkaline phosphatase activity in mycorrhizal root samples, and in addition we found an extra peak which appeared to be VAM-specific phosphatase. These findings suggest that the synthesis of acid and alkaline phosphatase dependent on the host species and the VAM fungus strain used.

The present study indicates that VAM fungal infection is not favourable to the host in the initial period of invasion and establishment. Figure 2b shows an initial decrease (10th day) in alkaline phosphatase activity in mycorrhizal roots, which may have been due to P components in the plant roots being trapped by the VAM fungus. It has been suggested that the VAM fungi depend on soluble organic metabolites in the root apoplast for growth and further colonization of the root (Schwab et al. 1991). Establishment of VAM fungi may well require about 20 days, after which the fungus increases the uptake and use of P by the host plant.

In the present study we extracted cytokinin from cowpea roots by a simple procedure using 0.1 *N* NaOH (pH 10) and ethyl ether (Vreman and Corse 1975). Figure 3 shows the elution pattern of cytokinin on high-performance liquid chromatography detected at 254 nm. The retention time for authentic cytokinin (Kinetin, Sigma Chemicals) and for the cytokinin extracted from the mycorrhizal and non-mycorrhizal cowpea roots was the same (4.7 min). The cytokinin content in the mycorrhizal roots was 23, 55, and 156% greater on the 15th, 30th, and 45th days, respectively, than in the non-mycorrhizal roots (Table 2). This significant increase in cytokinin content was directly related to the age of the plant and the percentage of VAM colonization.

In order to confirm a cytokinin increase in mycorrhizal roots we used a soybean callus tissue bioassay as described by Miller (1965). There was an increase of 146% in callus tissue weight for the extracts from mycorrhizal roots compared with the non-mycorrhizal roots on the 45th day (Fig. 4). This confirms that the VAM inoculation increased the synthesis of cytokinin in the cowpea roots.

Increased cytokinin concentrations (up to 111%) in mycorrhizal grass roots (Allen et al. 1980) and in VAM fungal spores (Barea and Azcon-Aguilar 1982) have been reported previously. The present study supports earlier findings that VAM fungi are involved in the synthesis of cytokinin in host roots. Cytokinins are known to enhance

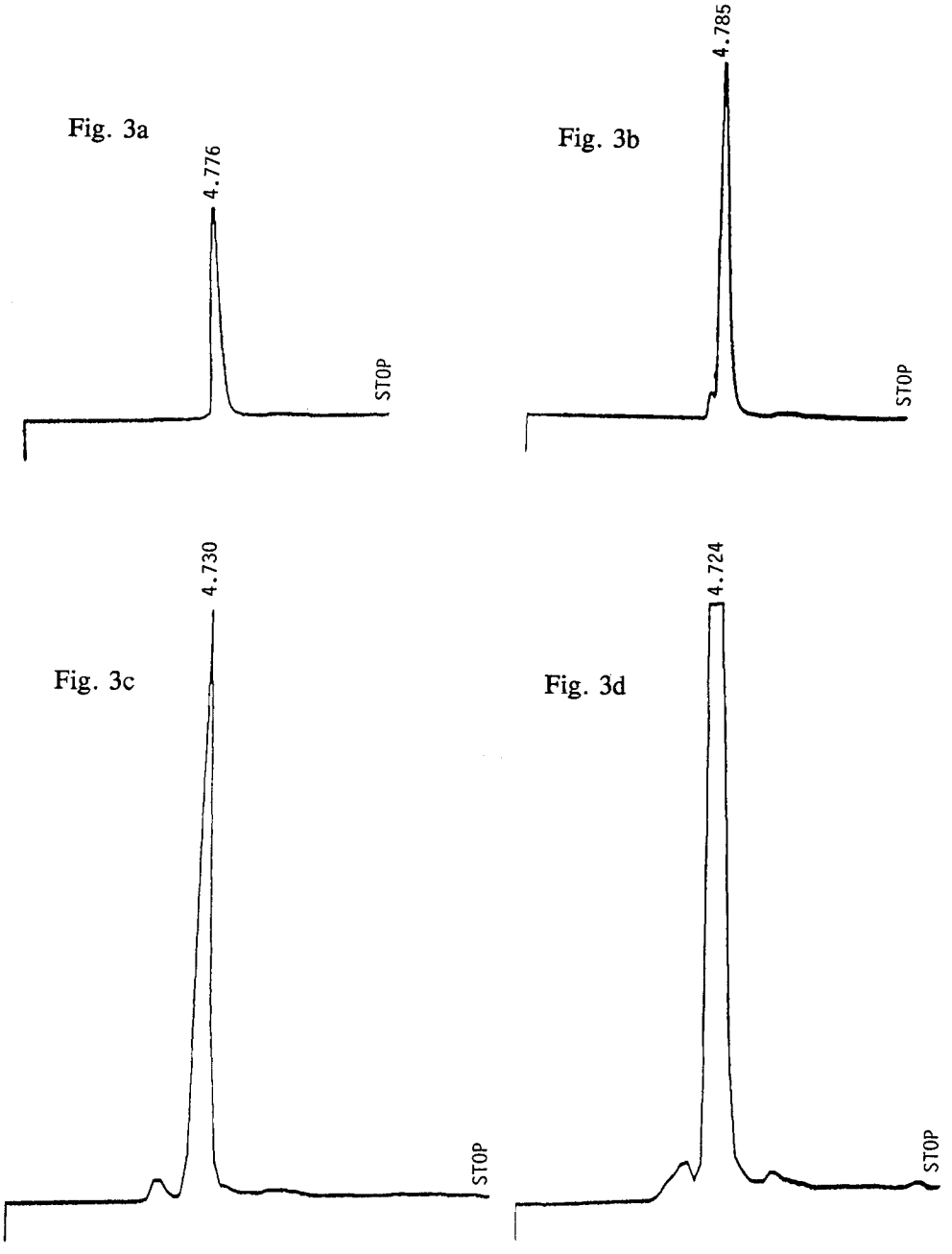


Fig. 3a–d Elution pattern from high-performance liquid chromatography column during analysis of an NaOH-ethyl ether extract of cytokinin from 30- and 40-day-old non-mycorrhizal (**a, c**) and mycorrhizal (**b, d**) cowpea roots, respectively. Sample injection: 20 µl; flow rate: 1 ml min^{-1}; solvent system: methanol : water (60 : 40); ultraviolet detection: 254 nm

Table 1 Influence of a vesicular-arbuscular (VAM) fungus (*Glomus pallidum*) on the growth, shoot P, and mycorrhizal colonization of cowpeas (*Vigna unguiculata*) in sterilized soil

Age of plant (days)	Shoot dry weight (mg $plant^{-1}$)		Shoot P (mg g^{-1})		VAM colonization (%)	
	NM	M	NM	M	NM	M
10	146 ± 10.6	132 ± 6.7	1.46 ± 0.02	1.20 ± 0.03	0	16.0 ± 1.2
20	203 ± 14.0	207 ± 12.1	1.60 ± 0.07	1.58 ± 0.08	0	28.8 ± 3.5
30	518 ± 13.9	593 ± 14.8	1.65 ± 0.06	1.97 ± 0.08	0	37.4 ± 5.9
40	925 ± 11.6	1081 ± 20.0	1.88 ± 0.07	2.24 ± 0.09	0	54.6 ± 6.8
LSD ($P = 0.05$)		64		0.23		

Means ± SE, $n = 5$ replicates. NM, Non-mycorrhizal plants; M, mycorrhizal plants; LSD, least significant difference

Table 2 Cytokinin concentration in mycorrhizal (M) and non-mycorrhizal (NM) cowpea roots at different stages of plant growth

Age of plant (days)	Cytokinin concentration ($\mu g\ g^{-1}$)		Colonization in mycorrhizal roots (%)
	NM	M	
15	0.52 ± 0.013	0.66 ± 0.016	23.4 ± 1.4
30	0.47 ± 0.011	0.73 ± 0.021	57.0 ± 4.7
45	0.84 ± 0.022	2.15 ± 0.064	78.6 ± 4.4
LSD ($P = 0.05$)	0.20		

For explanations, see Table 1

root growth and reduce resistance to microbial invasion of host tissue (Haberlach et al. 1978). Increases in the cytokinin content and phosphatase activity of cowpeas may explain the increased growth response mycorrhizal cowpea plants, are reported previously (Ames et al. 1991).

Acknowledgments This research was supported by a grant from the Postgraduate Board of the University of the West Indies, Mona. We thank Dr. H. N. Asemota and Mr. Odelly Henry for technical assistance in this study.

Fig. 4 Bioassay of cytokinin extracts from mycorrhizal and non-mycorrhizal cowpea roots at different stages of plant growth. 10-mg Soybean callus tissue pieces were initially inoculated to Miller's medium containing 100 µl cytokinin extract in 100 ml medium. VAM, Vesicular-arbuscular mycorrhizae

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