

## Effect of VA Mycorrhizae and Bark Ash on the Growth and N<sub>2</sub>-Fixation of Two Legumes

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### Abstract

Bark ash, which contains all the nutrients needed for plant growth except nitrogen, was compared with poorly soluble apatite and soluble superphosphate as P-source for two legumes grown in a soil of pH 4.9 and a P-content of 40 ppm Olsen-P. The ash addition raised the soil pH to 6, which was corrected for by adding lime to the other treatments. P was added at a rate of 64 ppm P (45 mg P/pot) in two experiments with common vetch (*Vicia sativa*) in steamed and in unsteamed soil, respectively, and one with field pea (*Pisum arvense*) in unsteamed soil. Half of the plants in each P-treatment and in the unfertilized control were inoculated with *Glomus* sp. E<sub>3</sub>. The solubility of P in bark ash was intermediate, as compared to apatite and superphosphate, and high enough for non-mycorrhizal plants to grow as well with ash as with superphosphate. In the control and apatite treatments mycorrhizal vetches had significantly more nodules and better growth than non-mycorrhizal ones. VAM-inoculated vetches had higher N<sub>2</sub>-fixation (acetylene reduction) rate and better growth both in steamed and in unsteamed soil, although the effect of inoculation was not significant in the presence of a natural VAM population. With field pea a significant increase in nodule biomass, N<sub>2</sub>-fixation, P-uptake and growth was obtained by combined VAM inoculation and ash addition. The results indicate that this was not only a P-effect but also a consequence of improved uptake of some micronutrients from the bark ash, and show that bark ash can be a good fertilizer for mycorrhizal legumes even at high soil-P levels.

Keywords: VA mycorrhizae, bark ash, phosphorus, legumes, N<sub>2</sub>-fixation

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## 1. Introduction

The beneficial effects of vesicular-arbuscular mycorrhizae (VAM) on nutrient uptake and growth of plants, especially under conditions of low phosphorus availability, are well documented (see Abbott and Robson, 1984; Gianinazzi-Pearson, 1986). Numerous studies have also shown that VA mycorrhizae stimulate nodulation and  $N_2$ -fixation of legumes, mainly due to improved host P-nutrition (see Bagyaraj, 1984; Hayman, 1986). There are also indications of more direct VAM fungus - *Rhizobium* interactions (Azcon-Aguilar et al., 1982; Bayne and Bethlenfalvay, 1987). VA mycorrhizae can also improve plant uptake of Cu, Zn, Fe, Mo and other nutrients (see Cooper, 1984) which are important for the nitrogen fixation process.

Apart from nitrogen, ash of plant origin contains all the macro- and micro-nutrients that are needed for plant growth, and it has traditionally been used in agriculture for improving soil fertility. In Finland large amounts of wood and bark ash are formed as a waste product of the wood processing industry, and occasional efforts have been made to find suitable ways of utilizing this ash as a fertilizer. Earlier studies in Finland and Sweden showed that tree growth on drained peatland was clearly stimulated by ash fertilization (Lukkala, 1951; Malmström, 1952). These studies, as well as later studies with legumes (Müller, 1983) indicated that the beneficial effect of ash was largely due to its phosphorus content. Malmström (1952) also made the observation that formation of ectomycorrhizae on trees was stimulated by the ash.

Many of the nutrients in ash are present as poorly soluble oxides, and thus not immediately available to plants. Studies with fly ash from coal-fired power stations indicated that the phosphorus compounds could not be utilized by plants (Adriano et al., 1978). Since VA mycorrhizae are known to improve utilization of poorly soluble P-sources (Cooper, 1984), it was interesting to see whether the presence of VA mycorrhizae would make ash nutrients more available to plant roots.

The objective of this study was to compare bark ash with apatite and superphosphate as P-source for VA mycorrhizal legumes, which are self-sufficient in nitrogen, the only nutrient not present in ash.

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Table 1. Nutrient content of bark ash

Nutrient	Total <sup>1</sup>	Extractable <sup>2</sup>	Solubility (%)
P mg/g	16.3	2.6	16
K	48.6	13.6	28
Ca	265	127	48
S	11.1		
Mg	34.4	5.9	17
Fe	12.5	0.09	0.7
Mn	13.3	1.0	8
Zn	0.3	0.1	37
Cr $\mu\text{g/g}$	28		
Cu	126	9.6 <sup>3</sup>	8
Mo	69	4.4 <sup>3</sup>	6
V	20		
B	249		
Co	74	9.7	13
Cd	10		

<sup>1</sup> 6 N HCl soluble (data provided by the Environmental Control Laboratory, Enso-Gutzeit Oy)

<sup>2</sup> 1 M NH<sub>4</sub>-acetate extractable (pH 4.65, extraction ratio 1:30)

<sup>3</sup> 0.2 M NH<sub>4</sub>-oxalate extractable (pH 3.3, extraction ratio 1:30).

## 2. Materials and Methods

### *Soil and fertilizers*

A loamy silt soil from an abandoned field, not cultivated for at least 15 years, was used in the experiments. The soil pH was 4.9, NaHCO<sub>3</sub>-extractable P 40 ppm and NH<sub>4</sub>-acetate-extractable P 14 ppm, total C 2.3% (w/w) and total N 0.2%.

Bark ash from a sawmill (Enso-Gutzeit Oy, Uimaharju) with a nutrient content as shown in Table 1, was used in the experiments.

The apatite was a hard magmatic rock phosphate (90% fluoroapatite) from the Siilinjärvi mine in Finland (Kemira Oy), finely ground (72% passing through a 100  $\mu\text{m}$  sieve) and containing 16.3% P, 38.8% Ca, 0.15% K and

Table 2. P-content and solubility of phosphorus in bark ash, apatite and superphosphate as estimated by alkaline and acid extraction

P-Source	<sup>1</sup> Total P mg/g	NaHCO <sub>3</sub> - <sup>2</sup> Soluble P µg/g	NH <sub>4</sub> -acetate- <sup>3</sup> Soluble P µg/g
Bark ash	16.3	1170	700
Apatite	163	13	86
Superphosphahte	82.9	3650	1290

<sup>1</sup> 6 N soluble

<sup>2</sup> pH 8.5, extraction ratio 1:20

<sup>3</sup> pH 4.65, extraction ratio 1:10

2.6% F (data provided by the Oulu Research Institute, Kemira Oy). The superphosphate (Kemira Oy) contained 8.7% P, 20% Ca and 12% S.

The amount of soluble P in the fertilizers was estimated by alkaline (Olsen et al., 1954) and acid (Vuorinen and Mäkitie, 1955) extractions (Table 2). The acid acetate extracted less P from bark ash and superphosphate than the alkali, while the reverse was true for apatite. This is in agreement with other studies on the effect of pH on the solubility of different P-sources (Haynes, 1982; Cabala-Rosand, 1982; Mosse et al., 1976).

#### Plants and inocula

Seeds of two annual forage legumes, common vetch (*Vicia sativa* L. cv. Lola) and field pea (*Pisum arvense* L. cv. Lysima) were germinated in moist sterile sand and after 7 to 10 days uniform seedlings were selected for transplanting into 800 ml pots. All seedlings were inoculated with *Rhizobium leguminosarum* (a commercial inoculant from Baljvåxlaboratoriet, Sweden) and half of each fertilizer treatment was inoculated with *Glomus* sp. E<sub>3</sub> (similar to *G. fasciculatum*, obtained from Rothamsted Experimental Station, UK). The VA mycorrhizal inoculum (2.5 g/pot) consisted of infected maize roots with adhering sand, hyphae and spores, it was placed in the planting hole of each pot and covered with a thin layer of soil. In order to ensure the presence of similar bacterial populations in all pots, a filtrate of the inoculum (Hayman and Mosse, 1971) was added to the non-inoculated plants in steamed soil.

There were 5 VAM inoculated and 5 uninoculated replicates in each fertilizer treatment, except in the experiment with steamed soil, where the

treatment (superphosphate +  $E_3$ ) was excluded due to lack of space in the growth chamber.

Cross-contamination via outgrowing roots was prevented by covering the drainage holes of the pots with a water-absorbing tissue.

#### *Soil treatments and growth conditions*

Three separate experiments were carried out, two with vetch in steamed and unsteamed soil, respectively, and one with pea in unsteamed soil. The fertilizer treatments were the same in all experiments, i.e. phosphorus was added as bark ash, apatite or superphosphate at a rate of 64 ppm total-P (45 mg P/pot), corresponding to ca. 50 kg P/ha. The ash addition (2.7 mg/pot, corresponding to ca. 3 tons/ha), raised the soil pH from 4.9 to 6. This liming effect was corrected for by adding  $CaCO_3$  to the other treatments, including the unfertilized control.

Prior to adding the fertilizers and lime, part of the soil was steamed for 1 hr on two subsequent days in order to eliminate the natural VA mycorrhizal population.

In the first experiment (vetch, steamed soil) the pots were kept in a growth chamber (56 klux, 18/6 hr day/night, 25/18°C, 70/90% rel. humidity) for 8 weeks. In the two other experiments the plants were kept in a growth room with 5.6 klux, 18/6 hr, 26/22°C and no humidity control, for 10 weeks (vetch, unsteamed soil) and 7 weeks (peas). The plants were watered regularly and given no extra fertilizer.

#### *Measurements*

The nitrogen fixation activity of the legumes was determined as the acetylene reduction (AR) activity of intact, potted plants (Lee and Yosida, 1977). The pots were enclosed in nylon-polyethylene bags (Vakuking PAE 20/70, Wipak, Finland; found gas tight to acetylene, ethylene and propane for up to 24 hr) and injected with 10% acetylene and 1 ml propane as internal standard. The plants were put back under light and the gas phase was sampled at intervals during a 6–8 hr incubation period, during which the AR-activity was linear. The gas samples were analyzed for ethylene and propane by standard gas chromatography.

Total plant dry weight was recorded in the first experiment, shoot dry weights in the other experiments. Plant N was determined after wet digestion according to Bremner (1965) and the P content was analyzed colorimetrically (Bartlett, 1959) after wet digestion.

Table 3. Effects of P-fertilizers and VAM-inoculation (*Glomus* sp.  $E_3$ ) on common vetch grown in steamed soil. Within a column, mean values ( $n=5$ ) not sharing a letter are significantly different at  $p=0.05$ .

Treatments	Total DM mg/pot	N <sub>2</sub> -fixation nmol C <sub>2</sub> H <sub>2</sub> /h per pot	N %	Nodulation (0-5)	VAM-infection % root length per pot
No P	552a	<20a	2.35a	1.4ab	0
No P + $E_3$	1251b	1100b	2.12a	4.0c	37
Bark ash	1316b	35a	1.97a	2.2ab	0
Bark ash + $E_3$	1414b	1400b	2.30a	3.8c	35
Apatite	613a	<20a	2.97a	0.8a	0
Apatite + $E_3$	1387b	710ab	2.11a	3.2c	43
Super-P	1417b	300ab	1.91a	2.6bc	0
Super-P + $E_3$	-	-	-	-	-

Fresh root samples were fixed in formalin:acetate:ethanol (1:1:18) for subsequent estimations of nodulation and VA mycorrhizal infection. The nodulation of vetch roots was estimated visually on an arbitrary 0 to 5 scale while nodule number and biomass (dry weight of excised nodules) per cm root length (as determined according to Newman, 1966), was recorded for pea.

The VA mycorrhizal infection was determined with the gridline-intersect method as a percentage of infected root length (Giovannetti and Mosse, 1980) after clearing and staining with 0.05% trypan blue in lactophenol (Phillips and Hayman, 1970). The accuracy of the method was  $\pm 2\%$  for vetch and  $\pm 4\%$  for pea roots (Giovannetti and Mosse, 1980).

### Statistical analyses

The data were analyzed by 2-way analysis of variance (ANOVA), followed by Tukey's mean value test. Because the super-P +  $E_3$  treatment was not included in the first experiment (vetch in steamed soil), the super-P treatment was excluded from the 2-way ANOVA and compared with the other uninoculated treatments by 1-way ANOVA.

## 3. Results

### Common vetch in steamed soil

Steaming of the soil effectively eliminated the indigenous VA mycorrhizal fungi, and vetches that had not been inoculated were consequently non-mycorrhizal (Table 3). Non-mycorrhizal plants given no P or apatite had

Table 4. Effects of P-fertilizers and VAM-inoculation (*Glomus* sp. E<sub>3</sub>) on common vetch grown in unsteamed soil. Within a column, mean values (n=5) not sharing a letter are significantly different at p=0.5.

Treatments	Shoot DM mg/pot	N <sub>2</sub> -fixation nmol C <sub>2</sub> H <sub>2</sub> /h per pot	N %	Nodulation (0-5)	VAM-infection % root length
No P	697a	250a	2.17a	1.8a	42a
No P + E <sub>3</sub>	866a	490a	2.14a	2.0a	55a
Bark ash	735a	490a	2.29a	2.5a	58a
Bark ash + E <sub>3</sub>	769a	750a	2.08a	2.6a	49a
Apatite	670a	260a	2.45a	2.2a	53a
Apatite + E <sub>3</sub>	899a	525a	2.19a	2.8a	47a
Super-P	755a	345a	2.30a	2.4a	37a
Super-P + E <sub>3</sub>	680a	680a	2.41a	1.6a	35a

significantly lower total plant dry weight than plants in any other treatment, whereas the mycorrhizal plants in these two treatments grew as well as plants given superphosphate or bark ash. Also non-mycorrhizal plants grew as well with ash as with superphosphate, and inoculation did not further enhance growth. P-addition *per se* had no effect on growth, as long as the plants were mycorrhizal. All treatments affected root and shoot growth in the same way (data not shown separately), and regardless of total plant size the shoot biomass was about 75% of the total dry matter.

In steamed soil inoculation significantly improved nodulation in all fertilizer treatments, and mycorrhizal plants had slightly more nodules than non-mycorrhizal plants given superphosphate. Addition of P in any form did not significantly improve nodule formation in non-mycorrhizal plants.

In the control, bark ash and apatite treatments the nitrogen fixation activity of non-mycorrhizal plants was significantly lower than that of mycorrhizal ones. Intermediate AR-activities were measured for mycorrhizal plants given apatite and non-mycorrhizal plants given superphosphate, but the differences from the other treatments were not significant.

Neither P-addition nor VAM inoculation had any significant effect on the N-concentration of the plant material, and the higher nitrogen fixation rates of inoculated plants only resulted in higher dry matter production in all treatments except with bark ash, where the yield was high despite a low AR-activity (Table 3).

Table 5. Effect of P-fertilizers and VAM-inoculation (*Glomus* sp.  $E_3$ ) on field pea grown in unsteamed soil. Within columns, mean values ( $n=5$ ) not sharing a letter are significantly different at  $p=0.05$ .

Treatments	Shoot DM mg/pot	N <sub>2</sub> -fixation nmol C <sub>2</sub> H <sub>2</sub> /h per pot	Nodulation $\mu$ g nodule per cm root	Nodule size $\mu$ g/nodule	P-uptake $\mu$ gP/shoot	VAM-Inf. %
No P	871a	165a	2.2a	34.7ab	2.18a	75a
No P + $E_3$	842a	165a	3.2a	29.7ab	2.25a	90a
Bark ash	986ab	1610b	4.5a	38.1ab	3.18ab	81a
Bark ash + $E_3$	1113b	2025b	11.2b	71.3b	3.43b	88a
Apatite	823a	135a	2.2a	31.1ab	2.71ab	75a
Apatite + $E_3$	826a	205a	2.6a	23.8a	2.38a	89a
Super-P	996ab	185a	4.2a	32.7ab	3.05ab	75a
Super-P + $E_3$	931ab	180a	3.3a	18.0a	2.87ab	81a

With indigenous VA mycorrhizal fungi present in the soil, inoculation caused a small but non-significant increase in nodulation, nitrogen fixation and shoot growth of common vetch in all treatments except superphosphate, where nodulation and growth was slightly decreased (Table 4). The natural VAM infection level was as high as in the inoculated plants. Typical  $E_3$ -infection with oval vesicles was observed in the inoculated plant roots, but the relative proportions of natural and introduced infection were not estimated.

Bark ash and superphosphate had slightly greater effect on the studied parameters than apatite, but again the differences were not significant.

#### *Field pea in unsteamed soil*

The VAM infection level of field pea was higher than that of common vetch, and inoculation only slightly increased it (Table 5). As for vetches, *Glomus* sp.  $E_3$  was present in the inoculated roots, but the relative amount of infection was not determined.

Addition of phosphorus as bark ash or superphosphate only slightly increased nodulation, P-uptake and growth of peas, while apatite had even less effect. The nitrogen fixation rate was not affected either, except in the bark ash treatment, where the AR-activity surprisingly was increased ten-fold, compared to the controls.

VAM inoculation had no significant effect on any of the studied parameters in the control, apatite and superphosphate treatments. However, combined



bark ash and  $E_3$ -inoculation resulted in more than twice as much nodule biomass as ash only. Also the AR-activity was higher, but since bark ash *per se* caused a high nitrogen fixation rate, this difference was not significant. The large nodule biomass was due to the average nodule size being 2 to 4 times larger in the combined ash +  $E_3$  treatment than in all other treatments. Presumably this was, at least partly, a consequence of the high P-uptake in this treatment (Table 5). Inoculation slightly decreased the nodule size in the other P-treatments. The P-concentration in the plant material (data not shown) was not affected by any of the treatments.

#### 4. Discussion

Nutrient availability, not only total concentrations in fertilizers, is important for plant growth and also regulates the microbial activity in the rhizosphere.

The solubility of phosphorus in bark ash was intermediate, as compared to poorly soluble apatite and soluble superphosphate (Table 2), and sufficiently high for non-mycorrhizal plants to grow as well with ash as with superphosphate (Table 3).

Many studies have shown that rock phosphate can be a good P-source for mycorrhizal plants grown in acid soils (Bagyaraj, 1984). Also in this study mycorrhizal vetches could utilize apatite-P when non-mycorrhizal vetches could not (Table 3), although this magmatic rock phosphate has a much lower solubility than the more commonly used sedimentary rock phosphate (Yli-Halla and Lumme, 1987).

The poor growth of non-mycorrhizal common vetch in the uninoculated control and apatite treatments (Table 3) clearly showed the VA mycorrhizal dependency of this legume under conditions of low P-availability, which agrees with studies on other legumes, e.g. sweet vetch (Azcon-Aguilar et al., 1986). However, because the soil apparently contained enough plant-available phosphorus to sustain adequate growth of mycorrhizal plants, there was no response to P added as ash or apatite (Tables 3 and 4) or as superphosphate (Table 4). Since no combined superphosphate +  $E_3$  treatment was included in the experiment with steamed soil, it is not known if maximal growth was obtained with bark ash as P-source. The amount of plant-available P in the experimental soil (40 ppm Olsen-P) has been reported to be at a level where plants seldom respond to P-fertilization (Clarke and Mosse, 1981; Schubert and Hayman, 1986).

Most plants in the experiment with unsteamed soil were smaller than the corresponding ones grown in steamed soil, probably due to the less favourable growth conditions. In unsteamed soil only uninoculated plants given no P or apatite were larger, due to the poor growth of the non-mycorrhizal plants with limited P-supply, emphasizing the importance of the symbiosis for adequate P-nutrition and growth.

The importance of VA mycorrhizae for nodule formation under less favourable P-conditions has been well documented (Bagyaraj, 1984), and was clearly seen in the control and apatite treatments of this study (Table 3). Mycorrhizal plants also fixed more nitrogen than non-mycorrhizal plants, regardless of P-addition. The high dry matter yield of non-mycorrhizal plants fertilized with bark ash, despite their low AR-activity and number of nodules, may have been a consequence of enhanced uptake of mineralized soil nitrogen. Addition of plant ash is known to stimulate general soil microbial activity and nitrogen mineralization (Weber et al., 1985).

The lack of response to inoculation in unsteamed soil was probably due to the large population of indigenous VA mycorrhizal fungi, which readily colonized the roots of common vetch and field pea, and perhaps also were better symbionts for these legumes than the introduced *Glomus* sp. E<sub>3</sub>. This was rather unexpected, considering that the original soil pH was more than one unit lower than the experimental soil pH and that liming has been shown to decrease the degree of colonization by indigenous VAM fungi (Kucey and Diab, 1984). On the other hand, *Glomus* sp. E<sub>3</sub> is adapted to more acid soil conditions, and has recently been shown to perform poorly at soil pH 6.8 (Koomen et al., 1987). There is, however, considerable variation in symbiotic effectiveness among mycorrhizal fungi (Bayne and Bethlenfalvay, 1987; Schubert and Hayman, 1986) and another inoculant fungus might have performed better than strain E<sub>3</sub>. Inoculation in presence of a natural inoculum can be successful, as shown by Barea et al. (1980), who obtained good response to inoculation of alfalfa in two phosphate-fixing, unsterile soils and by Kucey and Diab (1984), who used VAM strains isolated from alkaline soils to inoculate alfalfa grown in limed soils.

Bark ash slightly increased P-uptake and nodulation of naturally infected field peas, and significantly improved nitrogen fixation, although this did not result in significantly higher dry matter production (Table 5). When the roots were colonized at least partly by the inoculant fungus E<sub>3</sub>, the P-uptake of ash fertilized peas was further increased to a level slightly higher than that obtained by adding superphosphate. This may have been one reason for

the remarkable effect of combined inoculation and addition of ash on nodule formation, which resulted in a 5-fold increase in nodule biomass per cm root, as compared to the uninoculated control and apatite treatments. Pereira and Bliss (1987) have shown that both number and size of nodules is affected by the amount of available phosphorus, but phosphorus alone cannot explain the difference in nodule biomass since P-concentrations were not at all affected by ash addition and the P-uptake was not significantly different from that of plants in other treatments with smaller and fewer nodules (Table 5).

We therefore believe that the *Glomus* sp. E<sub>3</sub> was more efficient than the indigenous VAM fungi in taking up some micronutrient of importance for nodule formation, e.g. Mo, Fe, Cu or Zn. These elements are all present in bark ash (Table 1), and although not verified by plant analyses in our study, this hypothesis is supported by numerous reports on increased uptake of micronutrients following VAM inoculation (e.g. Kucey and Janzen, 1987).

In conclusion, this study shows that bark ash can be a good P-source for non-mycorrhizal legumes even at high soil-P levels, and that mycorrhizal legumes fertilized with ash can have better nodulation, nitrogen fixation, P-uptake and growth than plants given superphosphate. Whether this is a P-effect only or a combination with enhanced micronutrient uptake remains to be determined in a more detailed study with enough replicates to overcome the large variation within treatments. Proper attention should also be paid to the characteristics of the symbiotic partners and the effectiveness of the symbiosis in the experimental conditions chosen. Although results from greenhouse studies are liable to erroneous interpretations, e.g. if the importance of pot size for uptake of nutrients (Kucey and Janzen, 1987) is not recognized, this study indicates that ash of plant origin can be used to eliminate phosphorus and micronutrient deficiencies of plants, especially if these have efficient VA mycorrhizal symbionts in their roots. The favourable liming effect of ash, which is caused by its high content of Ca-compounds (Table 1), adds to its value as fertilizer in acid soils, but may be less favourable in neutral and alkaline soils.

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