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Creation of a non-mycorrhizal control for a bioassay of AM effectiveness

2. Benomyl application and soil sampling time

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Abstract The study aimed to determine the most appropriate soil sampling time as well as dose and application time of benomyl for assessing AM effectiveness in field soils in a bioassay in a growth chamber. AMF infectivity and AM effectiveness assessed using benomyl were compared at seven sampling times between the thaw and the autumn. The effect of benomyl dose and application time on mycorrhizal suppression and phytotoxicity in irradiated soil was studied. Doses of 10–100 mg kg^{-1} and application times 2 weeks before sowing, at sowing and 1 week after sowing were investigated. Various Finnish field soils with their indigenous AMF communities were used. The main test plant species was oil-seed flax (*Linum usitatissimum*). In a comparison of sampling time, barley (*Hordeum vulgare*) was also used and phytotoxicity was studied additionally on red clover (*Trifolium pratense*), barley and pea (*Pisum sativum*) mutants. Sampling in the spring after the thaw resulted in the highest infectivity and AM response and the clearest differences between soils with varying AM potential. No evidence of temporal variation in benomyl effectiveness on mycorrhiza was found. The dose of benomyl sufficient to create a control with suppressed mycorrhization was 20 mg per kg soil at target moisture incorporated in the soil. Plant growth reduction in irradiated soil was observed with benomyl application 1 week after sowing only with flax and red clover. The most effective application time for benomyl was immediately before sowing.

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Introduction

Suppressing the formation or functioning of AM is the only way to create a valid control for assessing total growth or nutrient uptake effects of AM, because variation of the carbon cost of symbiosis needs to be taken into account. The need to create a non-mycorrhizal control for mycorrhizal field communities is increased by the prevalent change in AM utilization strategy from regular inoculations to management of indigenous or introduced AMF populations in the field. There is also an increasing practical interest to complement chemical soil analyses by assessing biological contribution to field soil fertility, in order to improve relevance to sustainable agricultural practices.

In our comparison of alternative ways to create a non-mycorrhizal control, benomyl application turned out to be the most appropriate method (Kahiluoto et al. 1999). Fungicide comparisons commonly conclude that benomyl is most effective in suppressing AMF (e.g. Menge 1982; Dodd and Jeffries 1989; Perrin and Plenchette 1993; West et al. 1993), yet the fungicide effects may be modulated by the AMF community structure (Schreiner and Bethlenfalvay 1996). Benomyl (methyl-1-(butyl-carbamoyl)-2-benzimidazole) is enzymatically hydrolysed to the active compound carbendazim (methyl 2-benzimidazolecarbamate, MBC) within a few hours after addition (Helweg 1973a) and to 1-butyl isocyanate (BIC) (Tang et al. 1992). MBC inhibits the formation of microtubules in the nuclear spindle, thus hindering cell division and protein synthesis (Davidse and Waard 1984). BIC inhibits the fungal cutinase enzyme, decreasing the ability of the fungus to penetrate through the plant cell cuticula (Koller et al. 1982). Benomyl and MBC reduce the germination of propagules in soil (Bailey and Safir

1978), the length of colonized root (Boatman et al. 1978), the numbers of arbuscules and live internal and external hyphae as well as the area of the fungal-plant interface (Sukarno et al. 1993; Kling and Jakobsen 1997) and hyphal P transport (Boatman et al. 1978; Larsen et al. 1996; Kling and Jakobsen 1997).

The effect of benomyl on soil nutrients and microflora seems to be small (Helweg 1973b; van Faassen 1974; de Bertoldi et al. 1977; Habte 1997). A general fungistatic effect is seen but no disturbance of the most important species or the microbial balance of soil (Ponchet and Tramier 1971), and only exceptional changes in N availability (Kahiluoto et al. 1999). Repeated soil application of benomyl may also inhibit non-mycorrhizal fungi (West et al. 1993) or nematodes (Elamayem et al. 1978) in the rhizosphere. Benomyl does not affect *Rhizobium* or its function (Helweg 1973b; Heinonen-Tanski et al. 1982; Heinonen-Tanski and Turkki 1987; Mårtensson 1992; Sugavanam et al. 1994) which is important for the use of Fabaceae as test plants in conditions relevant to sustainable agriculture.

Phytotoxicity of benomyl is relatively weak and rare (Chase 1985; Paul et al. 1989; Sukarno et al. 1993). However, chlorosis or biomass reduction of susceptible plant species has been reported (e.g. Sarhan and Kiraly 1982; Naiki and Dixon 1987; Meunier and Verhoyen 1989; Mihuta-Grimm et al. 1990). The phytotoxicity agents are N,N′-dibutylurea (DBU) (Shilling et al. 1994), a product of the reaction between water and BIC (Moye et al. 1994), and obviously vapours of BIC (Aragaki et al. 1994). DBU probably inhibits photosynthesis and consequently growth of susceptible plants (Shilling et al. 1994). No phytotoxic effects of carbendazim on barley were observed by Singh and Ramachandran (1983). With pea, benomyl was phytotoxic even after storage of treated seeds (Sandhu 1989). We are not aware of any reports on the phytotoxicity of benomyl on flax, red clover and barley.

The success of benomyl in reducing AM formation or P uptake and growth response of plant hosts varies with the soil (Spokes et al. 1981) and fungal species (Trappe et al. 1984; Dodd and Jeffries 1989; Schreiner and Bethlenfalvay 1997) and also depends on the method, timing and rate of application. Successful modes of application have been incorporation of a benlate suspension into soil (Lu and Miller 1989; Habte 1997; Kahiluoto et al. 1999), sometimes even a soil drench (Udaiyan et al. 1995), especially for thin soil layers (Waters and Borovicz 1994), or immersing otherwise undisturbed soil cores in fungicide suspension (Merryweather and Fitter 1996). Mixing carbendazim evenly into the soil suppressed mycorrhization, whereas soil dressing with an equal concentration had no effect in the studies by Kling (personal communication).

The effect of pesticides on organisms is often dependent on the rate (Ekelund et al. 1994). The applied rates of the active ingredient giving satisfactory suppression of AM have varied in pots from 2.5 mg kg^{-1} (Bailey and Safir 1978) to 625 mg l^{-1} with additional repeated applications (Waters and Borovicz 1994) as a drench. The phytotoxicity of pesticides is also rate dependent. Benomyl has commonly been applied before sowing, but has been successful even after established infection (Hale and Sanders 1982; Schreiner and Bethlenfalvay 1996). The rate of decomposition of benomyl to compounds with no fungicidal effect increases with increasing humus content of soil (Helweg 1973a). Little information is available on the dependence of mycorrhizal infectivity or effectiveness on season. It is obvious that temporal variation in the amount, composition and vitality of AMF structures, in nutrient status and in other field soil characteristics will affect fungal infection, AM function and possibly also the effectiveness of benomyl.

The objective of the present study was to use benomyl to develop a method for creating a non-mycorrhizal control for assessing the AM effectiveness of field soil in a bioassay, in terms of crop growth and nutrient uptake. The most appropriate sampling time to get a clear and representative indication of AM effectiveness was determined, as well as the benomyl rate with no phytotoxicity giving maximal suppression of AM. The optimal benomyl application time and the dependence of the sampling time and benomyl rate on soil with its AMF community and the test plant were also investigated.

Materials and methods

Experiments

Experiment 1. Sampling time

Experiment 1 examined temporal variation in mycorrhizal infectivity and effectiveness and in the effect of benomyl on AMF, as well as the dependence of this variation on the host plant. Infectivity and effectiveness at seven sampling dates were compared using two host plants in three soils representing two soil types and climatic conditions, and three different management histories (Table 1). The plots were sampled once a month, starting in the spring from a frozen soil and ending just before the soil was frozen in the autumn. Benomyl was used to create a control with suppressed mycorrhization. The loam soil originated from treatments with 0 and 45 kg P ha^{-1} a^{-1} since 1977 in a long-term experiment in cereal rotation situated at the North Savo Research Station of the Agricultural Research Centre of Finland at Maaninka (63°09′N 27°19′E). The clay soil originated from a farm field with organic management since 1968 at Ypäjä (60°46′N 23°22′E). The weather conditions in the field during the experimental season were different at the two locations. The same 2.5 m × 4 m plots of one block of the field experiment and of the farm were sampled in 1995 repeatedly. Two adjacent plots with P fertilization histories different to the field experiment were chosen.

Soil samples consisting of five subsamples were taken from the plow layer (0–20 cm), avoiding previous sampling points. The soil mixtures were carefully prepared separately for each 1-l (7 × 26 cm) black PVC pot without drainage. Benomyl (a.i.) at 10 mg per kg soil at target moisture suspended in 50 g water (non-mycorrhizal control), or the same amount of plain water (mycorrhizal treatment) was incorporated with 1 l soil. The

Table 1 Treatments for the sampling time comparison (experiment 1). All other treatment combinations were included, but clay was excluded at the first sampling time (the codes used in the figures and tables are in parentheses)

Sampling date	Soil type and management history	Plant	Benomyl
3.4	Clay, organic farming (Clay)	Flax	None (Untreated)
15.5	Loam, 45 kg P ha^{-1} a^{-1} (Loam 45P)	Barley	10 mg kg^{-1} (Ben10)
15.6	Loam, 0 kg P ha^{-1} a^{-1} (Loam 0P)		
17.7			
17.8			
15.9			
16.10			

amount of benomyl was calculated for the soil at target moisture so as to achieve equal concentrations in the growing conditions. Equal soil volumes for different field plots and sampling times were used instead of equal dry weights to achieve greater relevance to the plant growth and nutrient uptake conditions in the field (Reganold and Palmer 1995). Each pot with soil from the same field plot and sampling time contained the same weight of dry soil. Water was added to 50–55% and 55–60% water-holding capacity for loam and clay, respectively.

Soil sampled before field fertilization (3 April and 5 May) was fertilized to simulate the nutrient status of the field soil at the start of the growing season, i.e. at the time of infection. Soil from experimental plots with no added P was mixed with 110 mg l^{-1} fertilizer (20% N, 15% K, 2% S, 1.5% Mg, 0.03% B, 0.0008% Se) (NK-lannos, Kemira Oy, Finland) and from plots with 45 kg P ha^{-1} a^{-1} also with 204 mg l^{-1} superphosphate (8.5% P, 20% Ca, 11% S) (Superfosfaatti, Kemira Oy) diluted in part of the added water, according to the annual use in the sampled field. At the second sampling date, there was an additional control treatment with no added P. The farm field was not fertilized during the experimental period. The soil properties in the field are presented in Table 2, and P and N availability in the pot soil at sowing is presented in Fig. 1c.

The test plants were flax (*Linum usitatissimum* L.) cv. Linetta (Deutsche Saatveredelung, Lippstadt-Bremen) and barley (*Hordeum vulgare* L.) cv. Arra (Agricultural Research Centre of Finland). Three pregerminated seeds were sown per pot. After emergence, they were thinned to one seedling per pot, retaining the tallest seedling. The pots of the treatments were organized in the growth chamber to four blocks so that each treatment occurred once in every block, at every sampling time. Within the blocks, the pairs of pots consisting of the same soil sample with and without benomyl were located next to each other. The pairs of pots were located at random and circulated daily. The growth chamber had artificial lighting by 36 W Gro-Lux Fluorescent Tubes (Sylvania, Germany), with a day length of 16 h and a temperature of 24/16 °C ± 0.5 °C. At the height of the seedlings the light intensity was 80–100 and at the top of harvested plants 135–170 μmol s^{-1} m^{-2}. The CO_2 concentration varied from 510 to 560 ppm at noon and the relative humidity was 55–65%. The pots were watered to the individual target weight three times a week. The experiments were harvested 28 days after sowing.

Experiment 2. Effective dose and application time

In experiment. 2, the effectiveness of five benomyl doses and three application times in suppressing AM was compared. The dependence of rate effect on soil with different humus contents and indigenous AMF populations, and on soil sampling time, was also studied (Table 3). The sampling times of spring and autumn were chosen on the basis of experiment 1 as most appropriate. Only the benomyl doses with were most potential in the spring were included in the autumn. Four 2.5 × 8 m blocks from the treatment with 0 kg P ha^{-1} a^{-1} of the long-term experiment utilized in experiment 1 were sampled on 2 April and 4 September 1996. Four blocks of 2.5 × 4 m from an organically managed field with a 3-year-old clover grass in Juva (60°53′N 27°53′E) were sampled on 15 April 1996. At the sampling in the spring, the soils were frozen and covered by snow. At the autumn sampling, the field was harvested but not yet cultivated. The soil was sampled as in experiment 1. The AMF communities of the two soils differed notably from each other according

Table 2 Soil properties at the start of the experiments. For treatment codes, see Table 1

	Soil texture			Organic matter		Exchangeable cations						
	<2 μm (%)	2–63 μm	>63 μm	pH_{CaCl_2}	(%)	Ca	K	Mg	P_{NaHCO_3}	P_{H_2O}	N_{NH_4+}	N_{NO_3}
						(meq 100 g^{-1})			(mg kg^{-1})		(mg kg^{-1})	
Experiment 1												
Clay	73	21	6	12	5.4	10	0.41	3.07	16	5.1	2.0	6.6
Loam 0P[a]	8	50	42	2.7	5.5	4.3	0.09	0.37	27	6.1	2.2	1.8
Loam 45P	8	50	42	2.7	5.5	5.1	0.09	0.39	70	20	0.9	0.5
Experiment 2												
Spring												
Maaninka	8	50	42	2.7	5.4	5.8	0.20	0.89	35	5.9	14	15
Juva	5	45	50	5.9	5.5	5.6	0.42	1.76	67	4.2	19	18
Autumn												
Maaninka	8	50	42	2.7	5.3	5.9	0.19	0.88	35	6.3	10	10
Experiment 3												
Spring												
Maaninka (irr.)	8	50	42	2.5	5.3	5.9	0.20	0.90	36	6.5	24	22
Autumn												
Maaninka (irr.)	8	50	42	2.5	5.3	5.9	0.19	0.86	36	5.5	29	11
Experiment 4												
Maaninka (irr.)	8	50	42	2.5	5.5	4.1	0.15	0.37	30	6.8	34	36

[a] Maanikan soil in experiments 2–4

Table 3 Treatments in the experiments on effective dose and application time (experiment 2) and on phytotoxicity (experiment 3)

Benomyl application		Experiment 2				Experiment 3			
	Rate (a.i.) (mg kg^{-1})	Spring Maaninka	Autumn Juva	Spring Maaninka	Autumn Juva	Flax	Clover	Flax	Clover
At sowing	0	+	+	+	–	+	+	+	+
	10	+	+	–	–	+	+	–	–
	20	+	+	+	–	+	+	+	+
	30	+	+	+	–	+	+	+	+
	50	+	+	+	–	+	+	+	–
	100	+	+	+	–	+	+	+	–
2 weeks before sowing	20	–	–	+	–	–	–	+	–
1 week after sowing	20	+	+	–	–	+	+	–	–

to the spore densities determined by centrifugation and sugar flotation (Väre et al. 1992). In the Maaninka soil, *Glomus mosseae* clearly dominated. In the Juva soil, the spore density was remarkably lower and the species composition was different from that in the Maaninka soil, with *Glomus hoi* and *Scutellospora calospora* as the most abundant species. The soil properties at sowing are presented in Table 2.

The soil mixtures were prepared and potted as at the two first sampling times in experiment 1, except the pots for one particular treatment, which were prepared 2 weeks before sowing and kept at +22 °C. Soil nutrient contents at sowing are presented in Table 2. Nine pregerminated seeds of flax cv. Linetta were sown per pot, the tallest three seedlings being retained after emergence. The pots were organized in the growth chamber to four blocks so that each treatment occurred once in every block. Within the blocks, the pots were located at random and circulated daily. The pots were kept in growing conditions similar to experiment 1, except that the temperature was 24 ± 1.5 °C / 17 ± 1.0 °C. For one particular experimental treatment, benomyl suspension was dressed from the top and injected through thin tubes 1 week after sowing. The tubes were set at three heights on two sides of the pot when filling the pot with the soil mixture. The pots were watered and harvested as in experiment 1.

Experiments 3 and 4. Phytotoxicity

In experiment 3, the phytotoxicity of the same benomyl treatments used in experiment 2 was studied with flax and red clover, which are appropriate test species for assaying the AM effectiveness of Nordic field soils (unpublished results). For red clover in the autumn, only the most promising doses of experiment 2 in the spring (Table 3) were applied. The same soil samples as in experiment 2 from the blocks of the treatment with 0 kg P ha^{-1} a^{-1} of the long-term experiment were used. The moist soil was irradiated with 10 kGy in 5-cm layers by Kolmi-Set Oy (Ilomantsi, Finland), left open for 1 week to detoxify and treated as in experiment 2 but with one-fifth lower fertilizer due to the nutrient flush caused by irradiation. Soil properties at sowing are presented in Table 2. Flax cv. Linetta and red clover (*Trifolium pratense*) cv. Bjursele (a local Swedish stock from the 19th century) were sown, thinned and grown as in experiment 2. Watering and harvesting were performed as in experiment 1.

Experiment 4 investigated the phytotoxicity of benomyl for two other potential test plants, barley cv. Arra and the mutant lines nod$^-$ myc$^+$ and nod$^-$ myc$^-$ of pea cv. Sparkle (see Kahiluoto et al. 1999). Benomyl (a.i.) rates of 0, 10 and 100 mg per kg soil at target moisture were compared. The soil was sampled on 15 September 1995 from one block of the same field treatment as in experiment 3. After irradiation, the soil was left to detoxify for 18 days, otherwise the experiment was performed as experiment 3.

Analyses

The soil pH was determined by 0.01 M $CaCl_2$ extraction (Ryti 1965), the content of plant-available soil P by water extraction (van der Paauw 1971) and by sodium bicarbonate extraction (Olsen et al. 1954), and exchangeable cations by 1 M ammonium acetate (Thomas 1982) and analysis by ICP. The soil mineral N content was determined from frozen (–18 °C) samples, extraction with 2 M KCl and measuring the N_{NH4+} and N_{NO3-} concentrations colorimetrically using a Skalar auto analyser (Linden 1981; Keeney and Nelson 1982).

The percentage root length colonized was measured at harvest. A representative sample of the root system was cleared and stained with methyl blue (Grace and Stribley 1991) and colonization was determined by the gridline intersect method (Giovannetti and Mosse 1980). In experiments 2 and 3, the hyphal length was also determined. The soil layer 5–6 cm below the soil surface in the pots was sampled and the method of Newman (1966) for estimating root length was used, as simplified by Tennant (1975) and applied by Jakobsen et al. (1992). The hyphae were stained with methyl blue. S & S filter paper circles with a mesh size < 2 μm (Schleicher & Schuell) were used with vacuum. Unstained, brown or septate hyphae, and those with a diameter < 5 μm (Bethlenfalvay and Ames 1987) were excluded.

The shoots and roots were cleaned and dried at 60 °C. Only shoots were sampled in experiment 1, because no clear root response to benomyl was shown in our previous study (Kahiluoto et al. 1999). Plant P, and in experiments 2 and 3 K and Cu contents at the later sampling time were analyzed by wet burning and ICP (Huang and Schulte 1985). The relative mycorrhizal effectiveness (RME), i.e. the mycorrhizal contribution to the growth (or nutrient uptake) of the mycorrhizal plant, is defined by the following formula: $RME\ (\%) = [(Y^{myc+} Y^{myc-}) / (Y^{myc+})] \times 100$ where Y^{myc+} and Y^{myc-} are the dry weights (or nutrient uptake rates) of the mycorrhizal treatment and the control with inhibited AM functioning, respectively.

Statistical methods

The effects of the seven sampling times (experiment 1) were studied on soil sampled from the three different field plots only, in order to examine descriptively whether there were temporal trends in common for all the field plots. Preliminary examination of the effects revealed that the spread of the distributions differed between the treatments. Further, the effects of the benomyl doses and application times on the distributions of the nutrient concentrations and nutrient uptake rates differed generally clearly in non-irradiated soil (experiment 2) with respect to location and/or spread. Additionally, in most cases, variances for some treatments were larger than for others with no consistency between the variables, and the differences between the treatments or lack of them were obvious. Correspondingly, in irradiated soil (experiment 3) the lack of a trend in response to dose,

or of other differences between the treatments, was so obvious that formal significance tests were unnecessary and in these cases the data were not modelled. The only exception was the effect of benomyl 20 mg kg^{-1} in irradiated soil (experiment 3) on shoot P uptake compared with no benomyl. The statistical significance of the difference was analyzed based on a split-plot model, where benomyl treatment, sampling time and their interaction were the fixed effects and block and its interactions with benomyl treatment, sampling time and benomyl treatment*sampling time were the random effects.Where the spread or location of the distribution or both were clearly different for some treatment from other treatments, that treatment was excluded from the modelling data. These cases are mentioned in the text or in Table 5 for the response variables presented there.

The two sampling times of experiments 2 and 3 were analyzed separately because only the most interesting spring treatments were included in the autumn. The same occurred also for the two test plant species of experiment 3 in the autumn, where only the most promising treatments were included for clover. The plant species of experiment 4 were modelled in two groups (flax and clover, barley and pea) due to the notable difference in the location and spread of the distribution of the response variables between these groups. Additionally, in experiment 3 shoot dry weight sampled in the spring was modelled separately for the two soils, and for the other soil shoot dry weight was modelled in two groups, because the spread of the distribution of the response variables was larger for some treatments than for others.

The experimental design of all the experiments was that of randomized complete blocks. The statistical analyses were based on the common mixed model for a randomized complete-block design. The models were fitted using the residual maximal likelihood (REML) estimation method. The accordance of the data with the distributional assumptions of the models was checked by graphic plots. The equality of the spreads across groups was assessed by the spread-level plot (SAS 1991), and the residuals were checked for normality using the box plot (Tukey 1977). The residuals were also plotted against the fitted values. Such a plot should have the appearance of a random scatter of points if the assumptions of the model are adequate. Planned comparisons between means were made by two-sided *t*-type tests or 95% confidence intervals (CI). To find out whether there was an effect related to dose or application time in non-irradiated soil (experiment 2), all the treatment effects were compared with that obtained by benomyl at 20 mg per kg soil at target moisture at sowing. This dose was the lowest one used in the autumn and at both sampling times and all application times. To examine whether any dose was phytotoxic, every dose was compared in irradiated soil with the no benomyl treatment (experiments 3 and 4). If the 95% CI does not include zero, the difference between means is statistically significant at the 5% level. The analyses were performed by the MIXED procedure of the SAS/STAT software (Littell et al. 1996). The statistical analyses of AMF colonization, i.e. the number of infected roots per 100 intersections, were performed as in our previous study (Kahiluoto et al. 1999).

Results

Some single observations are missing due to occasional technical difficulties. Some data include observations of unexplained discrepancy. Their influence on the results was examined by analysing the data with and without them. The influence was not substantial in any case and the whole data are presented.

Soil sampling time (experiment 1)

Infectivity and RME varied with soil sampling time. The most appropriate sampling time with the highest infectivity and the clearest differences in RME between soils with different AM potentials was the spring. The infectivity of the field AMF was highest in the soil sampled on 15 May immediately after the thaw and again at the end of the growing season, irrespective of the soil (Fig. 1a). Colonization in frozen soil sampled on 3 April (loam) was notably lower than in May or October and similar to the mid-summer. However, the effect of benomyl on root colonization, a measure of how representative are the results, showed no consistent temporal variation.

In contrast to infectivity, temporal variation in RME was dependent on the soil. In soils with higher AM effectiveness, variation in RME resembled that of infectivity, being the opposite of the soil with higher P level and lower mycorrhizal effectiveness (Fig. 1b). The variation was generally parallel for both host plants, but the differences between the sampling times were slightly higher for the more responsive flax than for barley. Temporal variation in RME in terms of shoot dry weight and shoot P uptake was similar. Yet, in terms of shoot P uptake, the estimates of RME were higher than in terms of shoot dry weight. Growth and P uptake as well as the effect on them of benomyl, and thus the absolute AM benefit, were clearly highest in the spring with a decreasing trend towards July and a small increase again in the autumn starting in August. This was true for all the soils and for both test plants in the comparison of sampling times (experiment 1); no clear difference between spring and autumn was observed in the dose comparison (experiment 2, Figs. 3, 4).

Loam P_{H2O} was stable throughout the whole season (Fig. 1c). In organically managed clay with a high humus content, the concentration was lowest in June after heavy rains but highest in mid-summer due to mineralization. Colonization and AM benefit were highest at the intermediate P availability of spring and autumn, due to very low extractable P. Soil concentration of soluble N varied little in organically managed clay. In loam, it was highest after the spring fertilization. No effect of benomyl on soil-soluble N was observed.

Benomyl effectiveness (experiment 2)

Effective suppression of mycorrhiza in this study was indicated by low AMF colonization, hyphal length, the concentration and uptake of P, Cu, K, and growth, as well as by a small spread in the distributions of these variables. For assessing AM effectiveness, the most important response variables are growth and P uptake. In all the variables indicating AM suppression, a notable decrease was achieved by benomyl (Figs. 2, 3, 4,

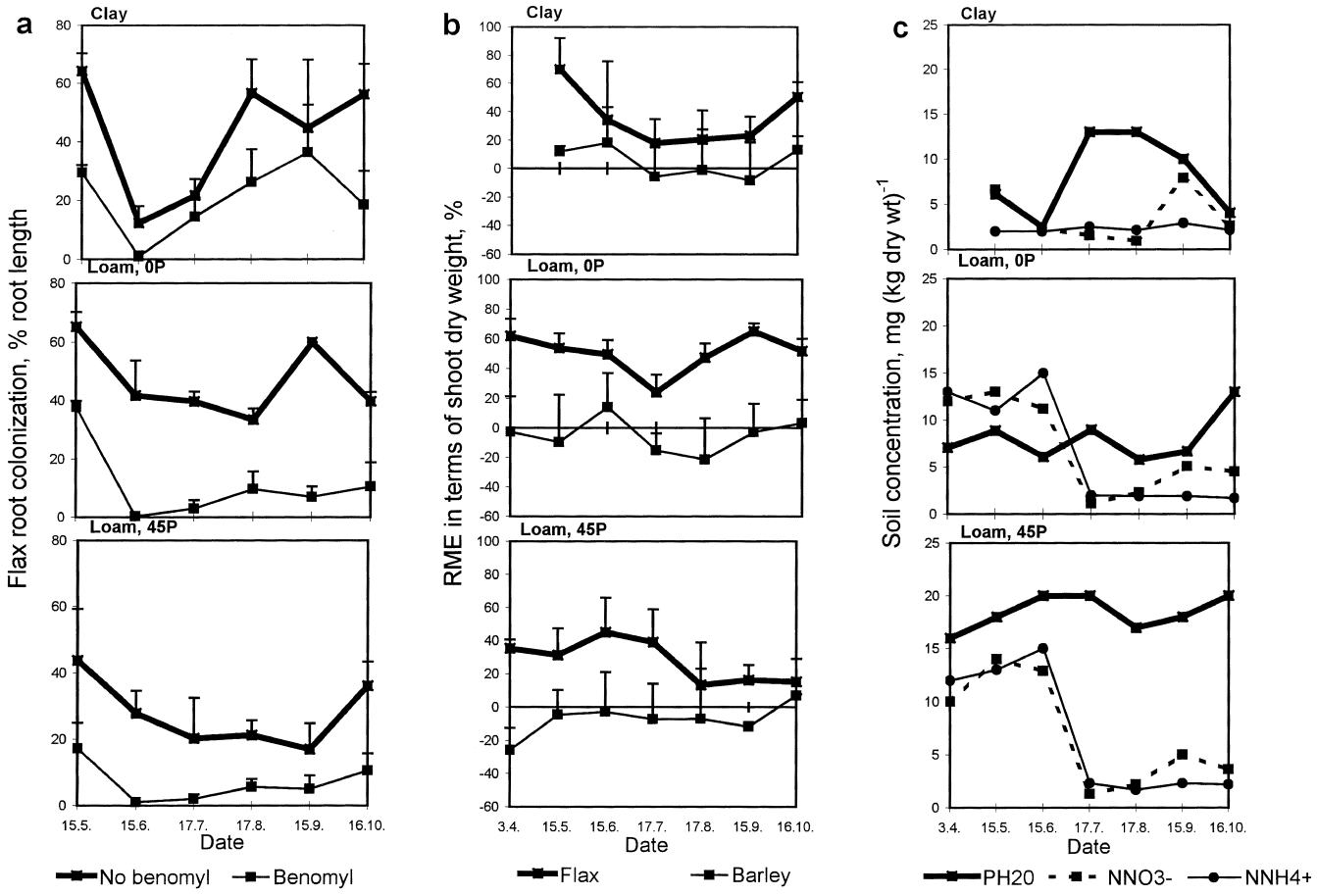

Fig. 1 Temporal variation in **a** AMF infectivity, **b** AM effectiveness and **c** soil concentration of P_{H2O}, N_{NO3-} and N_{NH4+} in experiment 1. RME above the zero line in *b* represents positive and under the line negative growth response to mycorrhiza. Values are means of four replicates in *a* and *b*, and results of one composite sample in *c*. For treatment codes, see Table 1; *bars* standard deviations

Table 4), except for root growth with no clear difference ($P > 0.09$) and root P concentration and uptake in the Maaninka soil in the spring. Nutrient contents were not modelled (see statistical methods). The percentage root length colonized in the Maaninka soil in the spring, however, was decreased by benomyl only slightly, but still clearly at some doses (the distributions for untreated soil and those for 30, 50 and 100 mg kg^{-1} did not overlap). In contrast to colonization, the hyphal length reduced by benomyl was the same in relation to the untreated soil at both sampling times, in accordance with the results of no temporal variation of benomyl effectiveness in experiment 1 (data presented for autumn only, Fig. 2). The hyphal length in the benomyl-treated soil (Fig. 2) did not differ from that in the irradiated soil (2.34 m g^{-1}, SD 0.72).

Effective dose depending on soil and sampling time

AM suppression by benomyl was dependent on the soil but no practically significant difference in dose requirement was observed. The effect of benomyl generally increased with increasing dose, but the effect of the dose was very small in comparison with the overall effect of benomyl. An improvement in the effectiveness of benomyl in trems of the lowest dose was obtained both in the Juva and Maaninka soils sampled in the spring, the lowest dose then being 10 mg kg^{-1}.

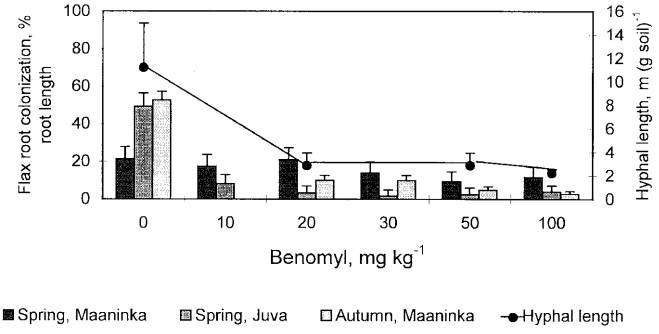

Fig. 2 AMF root colonization and hyphal length (Maaninka soil in the autumn) in non-irradiated soil (experiment 2). Values are means of four replicates; *bars* 95% confidence intervals for root colonization and standard deviations for hyphal length

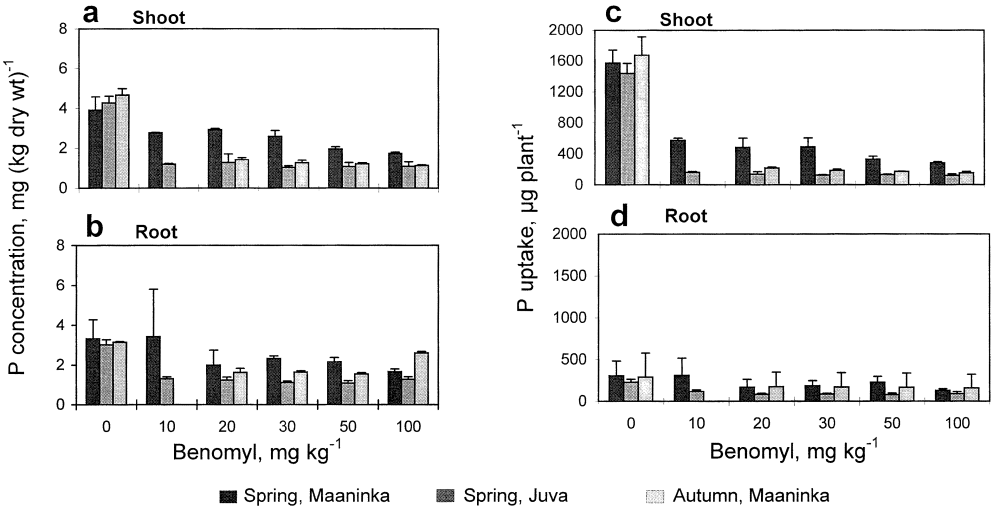

Fig. 3 Effect of benomyl dose on flax **a, b** P concentration and **c, d** P uptake in non-irradiated soil (experiment 2). Values are means of four replicates; *bars* standard deviations

Table 4 Results of analyses of variance and comparisons of doses for shoot dry weight (mg plant^{-1})

Non-irradiated soil (experiment 2)		Spring		Autumn
Benomyl dose		Maaninka	Juva	Maaninka
0–20 mg kg^{-1}	Difference	Distributions	Distributions	Distributions
	95% CI	clearly different	clearly different	clearly different
10–20 ”	Difference	Spread of [b]	+19 ($P=0.05$)	
	95% CI	distributions different	0, +38	
20–30 ”	Difference	−25 ($P=0.4$)	−12 ($P=0.21$)	+8 ($P=0.37$)
	95% CI	−96, +47	−31, +7	−10, +25
20–50 ”	Difference	+37 ($P=0.003$)[c]	−12 ($P=0.19$)	+9 ($P=0.27$)
	95% CI	+18, +57	−31, +7	−8, +27
20–100 ”	Difference	+45 ($P=0.001$)[d]	−0.0 ($P=0.98$)	+17 ($P=0.06$)
	95% CI	+26, +64	−19, +19	−1, +34
Application time at sowing – before/after[e]	Difference	+114 ($P<0.01$)	Distributions	+39 ($P<0.001$)
	95% CI	+42, +185	clearly different	+21, +56
Benomyl application		a) Maaninka $F_{2,6}=18.69$, $P=0.003$ [a, b]	$F_{4,12}=14.99$, $P<0.001$	
		b) Maaninka $F_{2,6}=8.31$, $P=0.02$		
		Juva $F_{4,12}=1.78$, $P=0.20$		
Irradiated soil (experiment 3)				
Benomyl dose		Spring	Autumn	Clover
0–10 mg kg^{-1}	Difference	−8 ($P=0.51$)		
	95% CI	−31, +16		
0–20 ”	Difference	+14 ($P=0.24$)	+9 ($P=0.41$)	+26 ($P=0.22$)
	95% CI	−10, +37	−13, +30	−21, +73
0–30 ”	Difference	−18 ($P=0.13$)	+10 ($P=0.33$)	−2 ($P=0.92$)
	95% CI	−42, +6	−11, +32	−49, +45
0–50 ”	Difference	−9 ($P=0.46$)	+4 ($P=0.67$)	
	95% CI	−32, +15	−17, +26	
0–100 ”	Difference	−0.0 ($P=0.99$)	+17 ($P=0.10$)	
	95% CI	−24, +23	−4, +39	
Application time at sowing – before/after[e]	Difference	−30 ($P=0.01$)	+3 ($P=0.74$)	
	95 CI	−54, −7	−18, +25	
Benomyl application		$F_{6,39}=6.18$, $P<0.001$	Flax $F_{5,15}=0.71$, $P=0.62$	Clover $F_{2,6}=1.33$, $P=0.33$[a]
Benomyl × crop		$F_{6,39}=0.71$, $P=0.64$		

[a] The soils/plants were modelled separately
[b] In the Maaninka soil in the spring, the variances for a) 20 and 30 mg kg^{-1} and for the application time, and on the other hand, for b) 10, 50 and 100 mg kg^{-1} were different so that the data were modelled separately within groups a) and b)
[c] Due to separate modelling of 20 and 50 mg kg^{-1}, comparison of 10–50 mg kg^{-1} was made instead of 20–50 mg kg^{-1}
[d] Due to separate modelling of 20 and 100 mg kg^{-1}, comparison of 10–100 mg kg^{-1} was made instead of 20–100 mg kg^{-1}
[e] Two weeks before sowing in the autumn, 1 week after sowing in the spring

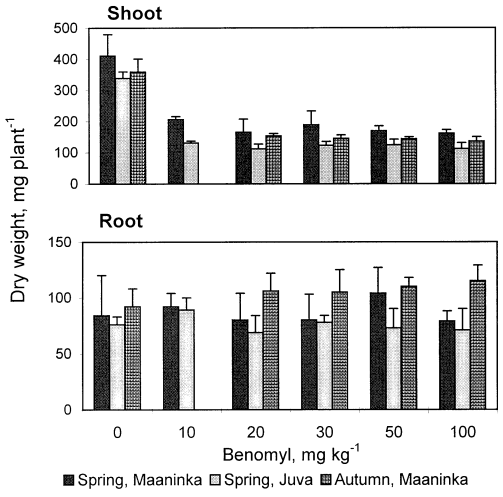

Fig. 4 Effect of benomyl dose on flax **a** shoot and **b** root growth in non-irradiated soil (experiment 2). Values are means of four replicates; *bars* standard deviations

The means for shoot growth and in the Maaninka soil, also for shoot P uptake decreased further with a dose of 20 mg kg^{-1}, but was accompanied by an increase in the spread of the distribution (Figs. 3c, 4a, Table 4). In the Juva soil, a further effect by 20 mg kg^{-1} was observed on root colonization ($P = 0.04$, $P > 0.35$ for higher doses compared with 20 mg kg^{-1}, Fig. 2). In the Maaninka soil, there was a further slight improvement by 50 mg kg^{-1} only on root colonization ($P < 0.005$, for treatment × soil interaction $F_{6,38} = 14.94$, $P < 0.001$, Fig. 2) and shoot P concentration (Fig. 3).

The dose requirement was not dependent on soil sampling time. Like in the Maaninka soil sampled in the spring, evidence of a slight but practically insignificant improvement in the effect of benomyl was observed with doses above 20 mg kg^{-1}, even if sampled in the autumn. The only other slight decrease was shown by benomyl 50 mg kg^{-1} in root colonization ($F_{3,9} = 13.44$, $P = 0.001$ for treatment effect, $P = 0.005$ for the difference between 20 and 50 mg kg^{-1}; no benomyl and application 2 weeks before sowing excluded from modelling due to obvious differences, Fig. 2) and shoot Cu concentration (3.05 v. 1.98 mg kg^{-1}, SD 0.30 v. 0.56, respectively; the distributions did not overlap). Yet, benomyl decreased colonization in the Maaninka soil more in the autumn than in the frozen soil sampled in the spring. At that sampling time, colonization was exceptionally low in both experiments 1 and 2 (Fig. 2).

There was no clear evidence of a difference in hyphal length between the benomyl doses ($P > 0.25$) (Fig. 2). The distributions for the treatments with no benomyl were so different from those for the other treatments that they were excluded from the modelling of the data. No dose effect occurred either in concentration and uptake of K. In P concentration and P uptake (Fig. 3), there were no differences either in the Juva soil in the spring or in the Maaninka soil in the autumn (lowest dose 20 mg kg^{-1}) (Fig. 3), nor in growth in the latter case (Fig. 4, Table 4).

Effective application time

The effectiveness of benomyl at 20 mg kg^{-1} applied at sowing was clearly better than when incorporated 2 weeks before sowing (studied in the autumn) or injected 1 week after sowing (studied in the spring). Root colonization, shoot P content and shoot growth were lowest if applied at sowing. The absolute differences between the means for colonization before and after sowing as compared with application at sowing (Fig. 2) were 15 and 12% ($P < 0.001$), respectively. The means for shoot P concentration and uptake before sowing differed by 1.89 mg kg^{-1} and 419 μg $plant^{-1}$ and those after sowing by 0.75 mg kg^{-1} and 546 μg $plant^{-1}$, respectively, from the mean at sowing in the Maaninka soil (Fig. 3). Shoot dry weight there was 39 mg $plant^{-1}$ higher with application before sowing and 113 mg $plant^{-1}$ higher after sowing compared with application at sowing (Fig. 4, Table 4). The differences of application times were even more clear in the Juva soil than in the Maaninka soil. One suspect outlier for colonization and shoot dry weight in the spring, obviously due to a technical failure in benomyl treatment after sowing, was deleted. There was a root response to the application time only in respect of P concentration and in P uptake in the Juva soil and in the Maaninka soil in the autumn. The response was similar to that of the other response variables.

There was no difference between application times in some response variables. In the Maaninka soil in the spring, with the weakest overall effect by benomyl, the spread of the distribution for root colonization was smaller at sowing than 1 week after, but with no evidence of a difference between the means ($P = 0.59$). In hyphal length, there was no clear difference in the autumn between application at sowing and 2 weeks before ($P > 0.30$). Generally there were no root effects.

Benomyl phytotoxicity (experiments 3 and 4)

No indication of phytotoxicity of any of the benomyl treatments on either of the test plants was observed in experiment 3, except for a decrease in shoot growth of 30 mg per flax plant and 58 mg per clover plant if injected 1 week after sowing (Fig. 5, Table 4). Although the data for nutrient content were not generally modelled due to an obvious lack of response to treatments (Fig. 6, for P) (see statistical methods), the lack of an effect by benomyl at 20 mg kg^{-1} on clover shoot P uptake and the absence of a dependence on sampling time was confirmed by statistical modelling ($P > 0.23$).

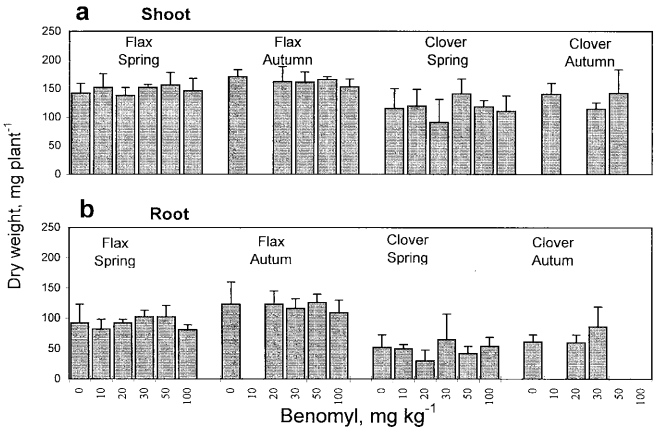

Fig. 5 Phytotoxicity of benomyl doses to flax and clover in terms of **a** shoot and **b** root growth in irradiated soil (experiment 3). Values are means of four replicates; *bars* standard deviations

In experiment 4, there was no indication of phytotoxicity by benomyl on barley or pea shoot growth ($P > 0.31$). There was, however, an interaction between effects of plant species and benomyl dose on their root dry weights ($F_{4,24} = 4.10$, $P = 0.01$) due to a difference between barley root dry weight with and without benomyl ($P < 0.001$), but with no difference for pea ($P > 0.31$). The difference between the means of barley root dry weight for no benomyl and 10 mg kg^{-1} benomyl (447 v. 329 mg $plant^{-1}$) was 118 mg $plant^{-1}$ (95% CI:+62, +174) with hardly any difference (8 mg) between the means for doses. There were no consistent differences in plant P concentration.

Irradiation to suppress mycorrhiza in experiments 3 and 4 was successful. Traces of AMF infection (<1%) were observed in only three pots, all in experiment 3, in soil sampled in the spring. Hyphal length, measured in experiment 3 only, was less than 22% of that in untreated soil at both sampling times (Fig. 2).

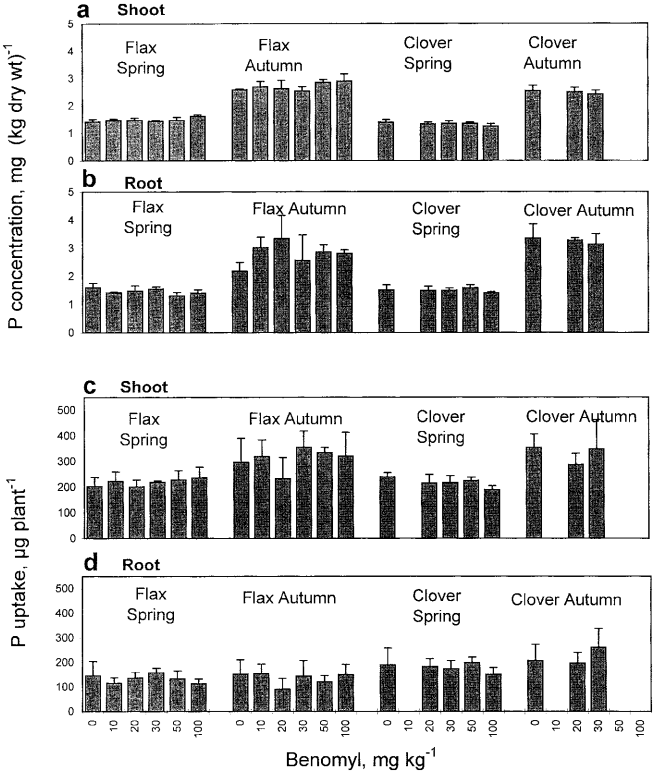

Fig. 6 Phytotoxicity of benomyl doses to flax and clover in terms of **a, b** P concentration and **c, d** P uptake in irradiated soil (experiment 3). Values are means of four replicates; *bars* standard deviations

Discussion

The success of benomyl in creating a non-mycorrhizal control was not dependent on soil sampling time. The decrease in colonization caused by benomyl did not vary between the sampling times, with the exception of the Maaninka soil in the spring, in the experiment on doses (experiment 2) sampled before the thaw and with a very low colonization in untreated soil. Even in that soil, a notable decrease in hyphal length and growth was produced by benomyl, similar to that in the same soil sampled in the autumn. These results indicate that the observed variation in effectiveness is due more to variation in mycorrhizal benefit than in benomyl effectiveness.

The most appropriate soil sampling time for assessing AM effectiveness in terms of growth or nutrient uptake was the spring before the start of the growing season. Infectivity was highest in the spring after the thaw and increased again towards the autumn, irrespective of the soil and management history. Temporal variation in AMF infectivity has been observed previously, e.g. by Sanders (1993). In soils with high AM potential, RME, estimated with benomyl, agreed with the variation in infectivity, except for the sampling time before the thaw where infectivity was low. Thus, RME was highest in the spring and again in the autumn for both plant species. In contrast, in soil with a high P level and low AM effectiveness, the temporal variation was the opposite, especially for flax. Obviously the heavy mycorrhization in the spring and autumn increased the carbon cost in relation to the lower benefit obtained. Therefore, in the summer the opposing patterns reduced the difference in RME between the soils with variable plant response to AM. The other possible sampling time would be the autumn, with infectivity and effectiveness close to that in the spring. Sampling in the spring, however, is more relevant to the conditions of infection in the field.

A possible explanation for the variation in infectivity is variation in the density of vital AMF propagules. This could well be highest in the spring, before the start of biological activity causing injuries and decomposition, and again in the autumn when new spores

and hyphae have been produced. AMF spore viability in field soils varied between the stages of the cropping season, having the same pattern in various soils in the study of Anwar and Jalaluddin (1998), and hyphal length peaked in the spring and autumn in Canadian forest soil (Klironomos and Kendrick 1995). AMF hyphae can survive winter freezing and remain infective (Addy et al. 1994). A contribution by seasonal variation in the dominant AMF species can not be excluded. Only in clay, with the clearest variation, is the effect probably strengthened by the observed variation in soil P availability. This is supported by parallel but even higher variation in RME in terms of P uptake than in terms of growth.

The inhibiting effect of benomyl on AM generally increased with increasing dose, but the effect of the dose was very small in comparison with the successful overall effect of benomyl. Benomyl caused a considerable decrease in all response variables which are well-established indicators of AM function. Hyphal length in the benomyl-treated soil did not differ from that in the irradiated soil. No phytotoxicity was observed at any benomyl dose applied at sowing to any of the plants tested, despite the growth suppression of barley roots at 10 mg kg^{-1} with no further effect at 100 mg kg^{-1}. Barley is a potential test plant only at exceptionally low P levels. Even if barley is used, this effect is not of importance because the root response is generally very inconsistent (Kahiluoto et al. 1999) and can, therefore, be excluded when assessing effectiveness. The optimal dose was thus that above which there was no practically significant increase in effect.

The most appropriate benomyl dose for assessing AM effectiveness was 20 mg per kg soil at target moisture, i.e. 25 mg per kg dry soil on average, irrespective of the soil and sampling time, although the improvement compared with 10 mg kg^{-1} was not drastic. Doubling the smallest dose studied, 10 mg kg^{-1}, strengthened the effect by decreasing the means for shoot growth and P uptake, which are essential in assessing AM effectiveness. This is in agreement with the results of Schreiner and Bethlenfalvay (1997), who showed that benomyl at 20 mg per kg air-dry soil inhibited spore germination by all three AMF isolates tested in contrast to 10 mg kg^{-1}, which had variable effects on the isolates. Only in the Maaninka loam and only at one of the two sampling times, in the spring before the thaw, was the effect of benomyl on mycorrhiza slightly improved compared with 20 mg kg^{-1} by increasing the dose. Even then, the advantage was mainly in the spread of the distributions and achieved with doses not smaller than 50 or 100 mg kg^{-1}.

Incorporation of benomyl at 20 mg kg^{-1} immediately before sowing was clearly the most effective application time in suppressing AM, with no phytotoxicity. Benomyl incorporation 2 weeks before sowing was more effective than 1 week after sowing. Benomyl after sowing did not suppress AM sufficiently, obviously due to uneven penetration of benomyl in soil when not incorporated, as suggested by the high variation of response. Early initiation of AM function before benomyl application may have contributed to the result. Injection 1 week after sowing was also the only application time with an obvious phytotoxic effect, presumably due to direct exposure of roots to benomyl suspension. In contrast to this study, 1 month incubation of benomyl at 10 mg kg^{-1} had no effect in our earlier work, in the same soil sampled at the same time of the season (Kahiluoto et al. 1999). The difference is obviously due to the difference in dose, because the relative effect of the lower benomyl dose of the former study was equal to that with incubation in the latter study. Due to lack of phytotoxicity by incubation and rapid transformation to the effective compound MBC after addition to the soil, the effect of incubation depends on the rate of decomposition (Helweg 1973a) and perhaps also on the adsorption to soil particles (van Faassen 1974).

Hyphal length correlated better than colonization level with the effect of benomyl on growth and P uptake. When incorporated into soil, benomyl may mainly affect the extramatrical hyphae directly while, due to incomplete elimination of infection, internal colonization protected by the plant tissue is not always reduced to the same extent. Therefore, a notable growth reduction was observed despite relatively high root colonization or no change in it in the present study in the comparison of sampling times in the spring and autumn (experiment 1), in the comparison of doses in the Maaninka loam in the spring with benomyl 10 and 20 mg kg^{-1} (experiment 2), and in our previous studies with benomyl at 10 mg kg^{-1} (Kahiluoto et al. 1999) and several earlier studies (e.g. Bailey and Safir 1978).

The results obtained were obviously not obscured by the effect of benomyl on soil nutrient status or pathogens. No change of soil P or N status by benomyl at 10 mg kg^{-1} was observed at any sampling time and an effect of dose on soil nutrient status is improbable (van Faassen 1974; Kahiluoto et al. 1999). There were no signs of pathogen attack and the main test plant flax is rarely attacked by root pathogens in Finnish field soils (Lehtinen 1998). The results can probably be applied also to somewhat longer growth periods than the 4 weeks used here. This is due to the significance of early infection and supported by the results of Habte (1997), who concluded that benomyl creates mycorrhiza-free conditions even up to 90 days at 25 mg per kg air-dry soil. Dependence of the effectiveness of benomyl on the sampling time, dose and application time did not vary notably between the soils studied, which were chosen to represent soil types and soil conditions from various geographical areas of Finland. The results may thus be applicable to most North European field soils and even to other field soils experiencing a thaw.

In conclusion, for assessing mycorrhizal infectivity and effectiveness of field soils, it appeared advisable to sample the soil in the spring after the thaw and to create a control with suppressed mycorrhization in bioassays of 4 weeks or slightly longer using 20 mg per kg soil in target moisture of benomyl incorporated into the soil immediately before sowing.

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