

CONTROL OF INK DISEASE BY TRUNK INJECTION OF POTASSIUM PHOSPHITE

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SUMMARY

Potassium phosphite was applied to 4-year-old chestnut plants either in the form of a foliar spray or by xylem injection to control the development of ink disease, following inoculation with *Phytophthora cinnamomi*. Foliar spraying reduced symptom expression by ca. 90% within 30 days, and later served to inhibit fungal colonisation of the stem. The injection treatment fully prevented infection, but the efficacy of the curative treatment depended on the initial level of disease severity. Potassium phosphite was injected annually into the trunks of a set of old chestnut and young walnut trees, naturally infected by, respectively, *P. cambivora* and *P. cinnamomi*. The treatment was ineffective on heavily diseased trees, but acted curatively on less infected ones. Two fungicidal applications on chestnut (one on walnut) were sufficient to promote plant recovery, as observed by improved vigour of the sprouts, larger size and deeper colour of the leaves, normal fruit development and ripening, a halt in the flux of ink, and the dehydration and compartmentalization of disease lesions.

Key words: *Phytophthora cambivora*, *Phytophthora cinnamomi*, MCE, foliar spraying, chestnut, walnut.

INTRODUCTION

Up until the middle of the 20th century, the chestnut (*Castanea sativa* Miller) was of particular importance to the economy of some mountain communities in Italy. Since this time, its importance has gradually declined, leading in some localities to substantial depopulation. Many of the chestnut trees in these areas have in the meanwhile succumbed to the ink disease, caused by *Phytophthora cambivora* (Petri) Buisman (Petri, 1917) and *Cryphonectria parasitica* (Murr.) Barr (Biraghi, 1947) (chestnut blight). Starting in the 1980s, there has

been a renewed demand for chestnut timber and nuts, complemented by active marketing efforts and the development of mechanised harvesting. These have acted together to restore the economic viability of chestnut growing. Stands of chestnut are also viewed as an integral component of the mountain scenery, and thus their conservation is important to maintain biodiversity and for the tourist industry. After a relatively long period of stasis, ink disease is once more on the rise in Italy (Vanini and Vettraino, 2001; Turchetti *et al.*, 2003). In Piedmont (NW Italy), the disease is widespread in the Pellice valley, Mongia, Pesio and Vermenagna. It is only patchily present in the Susa valley, Sangone and Grana, where most frequently, it is caused by *P. cambivora*, although a single case in which *P. cinnamomi* was implicated has been documented in a nursery plant growing in Chiusa Pesio (Cuneo).

P. cinnamomi is adapted to regions characterised by warm winters and compacted soils (Fonseca *et al.*, 2004; Vettraino *et al.*, 2005). In Piedmont it is the causal agent of root and foot rot in walnut (*Juglans regia* L.) (Belisario, 2005; Belisario *et al.*, 2006; Belisario *et al.*, 2007). The disease, in both chestnut and walnut, is spread by the movement of inoculum along country roads and the surface drainage net. Currently, disease control requires accurate diagnosis, and careful water management where environmental conditions are conducive to disease spread. Phosphorous acid salts, formulated as Al-ethyl phosphite (or phosethyl-Al), have been documented as being effective against *Oomycetes* diseases since 1977 in some horticultural crops (Bertrand *et al.*, 1977; Molot and Beyries, 1977; Chalandon *et al.*, 1980), and a little later in both citrus (Laville, 1979; Davis, 1982; Laville and Chalandon, 1982) and other woody plants (Darvas *et al.*, 1984; Fenn and Coffey, 1984). The biological activity of phosethyl-Al appears to be largely due to phosphorous acid (Reuveni, 1997; Zahid *et al.*, 2000; Daniel and Guest, 2005). On herbaceous plants and shrubs, foliar spraying or soil drenching with fungicide are simple and effective, but for a tree, plant height, fluctuation in wind direction and both the distribution of the roots and volume of soil they explore make these approaches untenable. Painting the trunk with fungicide can control localised infection on young trees

Table 1. Information on trees chosen for treatment with potassium phosphite (0.77 g H₃PO₃, cm⁻¹ of trunk diameter at 150 cm above the ground) by the MCE method, and the treatment schedule.

Location	Plant species	Trunk Ø (cm)	N° plants	1° treatment		Subsequent treatments (years)
				Symptom severity	Year	
Scagnello*	Chestnut	35-40	4	5	2002	--
			5	4		2003
Robilante*	Chestnut	40-50	3	5	2002	--
			3	4		2003
Torre Pellice A	Chestnut	29-31	1	5	2002	2003-2008
			1	4		
			2	5		
Torre Pellice B	Chestnut	140-165	3**	4	2004	2005-2008
			1	3		2005-2008
			2	1		2005-2008
Villar Pellice	Chestnut	63-128	1	4	2004	2005-2008
			5	1		
Villar Focchiardo	Chestnut	15-47	4	3	2006	2007-2008
			2	4		
Boves	Chestnut	21-58	2	3	2005	2006-2008
			1	2		
			3	1		
			2	0		
			1	5		
Viola	Chestnut	35-55	12	3	2004	--
			1	2		2005-2008
Luserna S. Giovanni	Walnut	11-17	9	4	2005	2006-2008
			4	4	2006	
			3	3	2006	2007-2008
			2	2	2006	

Experiments were stopped in 2004: *on all trees owing to conversion to organic production; **on two trees felled by a storm.

(Davis, 1982; Browne and Viveros, 2005), but this method may be ineffective for the elimination of root infections in mature trees. In avocado, *P. cinnamomi* infection was controllable by injecting fungicide through holes drilled in the trunk (Darvas *et al.*, 1984). Based on this experience, we have evaluated here the effectiveness of potassium phosphite infusion into the trunks of both chestnut and walnut to control ink disease in artificially inoculated and naturally diseased trees.

MATERIALS AND METHODS

Pot trials. Three-year-old chestnut plants of the variety 'Marrone di Chiusa Pesio', were transplanted into ten-litre pots a year before the experiments, and the plants were maintained in a nursery, reaching a diameter of 2-2.5 cm. A highly virulent *P. cinnamomi* isolate, obtained from a chestnut tree growing in Chiusa Pesio, was cultured at 25°C on agar medium containing V8 vegetable broth. The fungus was inoculated into the stem 12-15 cm above soil level by replacing a 6mm bark disc with an equivalent culture disc; in the uninoculated control plants a sterile substrate disc was used. To prevent desiccation, the bark which had been removed was

placed back over the hole, and sealed with parafilm.

Twenty-eight g of anhydrous phosphorous acid (98.5% H₃PO₃, M=82 gmol⁻¹, Sigma-Aldrich) were dissolved in 1 l of deionised water, and brought to pH 6.4 with 28 gl⁻¹ potassium hydroxide (98.5% KOH, M=56.11 gmol⁻¹, AppliChem GmbH) obtaining a solution of potassium phosphite for incomplete saturation of the acid (Ann, 2001). This solution was applied either as a foliar spray [1.2 gl⁻¹ active ingredient (a.i.) H₃PO₃] or as an injection (0.77 g a.i. per cm stem diameter) at 4-5 cm above the soil. This was achieved by infusion through a 200 µl pipette tip connected to a reservoir via a hole (2 mm diameter, 6-7 mm depth) drilled near the plant base.

Each treatment was carried out on 12 plants (3 replicates of 4 plants) arranged in a randomized block design, for a total of 84 plants as follows: (i) inoculated and sprayed four times (12 days before inoculation, 2, 16 and 30 days post-inoculation (dpi) either with water (control, 12 plants) or K phosphite (12 plants); (ii) inoculated and infused with water (control, 12 plants) or K phosphite either 12 days before inoculation (12 plants) or 14 dpi (12 plants); (iii) uninoculated and either sprayed (12 plants) or infused (12 plants) with water. Previous assays had shown no sign of phytotoxicity due

to spraying or injection of potassium phosphite solutions, so uninoculated controls treated with the fungicide were not included. The trial was repeated once. Treatment effects were monitored at 30 and 60 dpi, by measurement of lesion length. All data were subjected to variance analysis and the means compared with Tukey's test using Statistica software (StatSoft Inc., USA).

Field trials. Chestnut and walnut trees were assessed for their reaction to, respectively, *P. cambivora* and *P. cinnamomi*; diseased plants showed reduction in growth, the development of sparse, under-sized chlorotic leaves, small husks/fruits, branch death, visible ink exudation from the collar or along the trunk and the smell of fermented tannin. Field locations, tree species, trunk diameter, number of plants and disease severity at the time of the first treatment, and treatment timing, are provided in Table 1.

The chestnut samples were restricted to a small number of plants due to the reduced areas of each field, and all diseased plants were treated. The difference in disease severity between the first treatment and that of successive years was used to assess treatment effectiveness. The walnut samples included 24 9-year-old plants, with the disease being rather homogeneously distributed. Only the 9 walnut trees showing the most severe symptoms were treated in the first year, leaving 15 untreated ones; in the second year, nine of the 15 trees were treated, leaving 6 untreated.

Potassium phosphite was given using the Corradi-Ecoiatros infusion method (MCE), which is founded on fungicide release under atmospheric pressure. Three to six holes, depending on the size of the tree, each 3.5 mm in diameter and 40 mm deep, were drilled at the base of each tree into the active xylem; a 200 µl pipette tip was connected to each of 3 input tubes of a 1000 ml plastic bag which was hanged on the trunk about 3 m above the ground; all air present was eliminated from both the hole and the input tube to avoid the formation of bubbles. Following the information reported by Fernandez-Escobar *et al.* (1999), we measured the trunk diameter at 1.5 m above the ground, and estimated 0.77 g H₃PO₃ per cm trunk diameter to be the appropriate fungicide dose. Treatments were carried out in June-July, a period of reliable sunshine. On both tree species, disease severity was evaluated annually in July, except in 2008, when it was done both in July, to evaluate the effects of 2007 treatments, and in September, to evaluate those of the last treatments. An arbitrary disease index was scored on a scale ranging from 0 (healthy plant) to 6 (dead or non-recoverable plants) through the following intermediate levels: 1 = the appearance of sparse, undersized and chlorotic leaves; 2 = frequent undersized and chlorotic leaves; 3 = >25% of the canopy consisting of under-sized and chlorotic leaves, along with

some twig death and the first sign of ink efflux; 4 = 50% of the canopy showing symptoms, along with abundant ink efflux; 5 = >50% of the canopy with symptoms, abundant ink efflux along the trunk. Disease scores were converted into radians [$\theta = (\arcsin \sqrt{p}) \times 180/\pi$], subjected to variance analysis and the means compared with least significant difference (LSD) test using Statistica software (StatSoft Inc., Tulsa, Oklahoma).

RESULTS

Pot trials: foliar sprays and injections. At 30 dpi, *P. cinnamomi*, necrotic lesions were 135-245 mm in length (mean 202.8 mm) on untreated plants, and 17-60 mm (mean 26.2 mm) on the potassium phosphite sprayed ones. No lesions were observed on any uninoculated plants. By 60 dpi, two thirds of the control plants had died, and lesions on the survivors had a mean length of 247 mm. In contrast, on treated plants, the mean length of lesions had not increased, and these were largely isolated by the development of surrounding wound reaction tissue, as also occurred on the uninoculated plants. A similar result was obtained from the second experiment, in which control plants suffered 60% mortality, while lesion length in the potassium phosphite sprayed plants varied from 12 to 50 mm.

Plants injected preventively regularly absorbed the

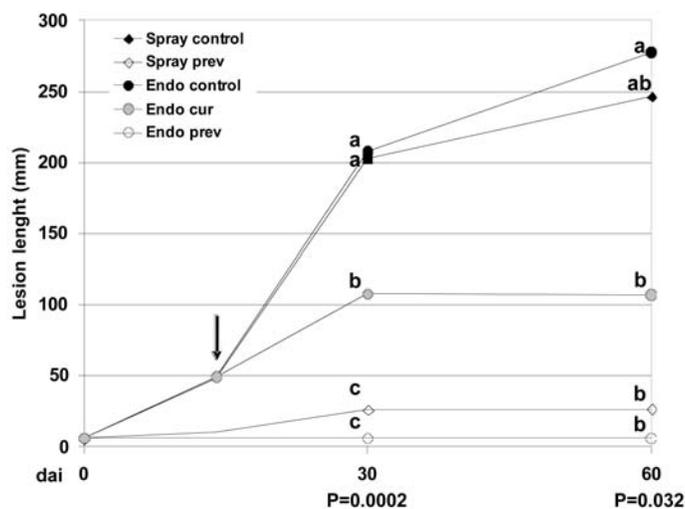


Fig. 1. *Phytophthora cinnamomi* lesion length (mm) 30 and 60 dpi. Stems of 4-year-old chestnut plants were either not treated, sprayed with a solution of potassium phosphite (four applications, starting 12 days before inoculation), or stem injected (one preventive application 12 days before inoculation, or one curative application at 14 dpi, shown by an arrow). The length of the original wound was not subtracted from final measurements. Uninoculated controls are not shown for better clarity. Statistical analysis was computed 30 and 60 dpi: means with a same letter are not statistically different following Tukey's test ($P = 0.0002$ at 30; $P = 0.032$ at 60 dpi).

fungicide and showed no visible toxicity symptoms. By 30 dpi, no disease symptoms were noted, although a wound response was apparent leading to complete or almost complete healing 60 dpi, as in the uninoculated plants.

Plants were treated curatively, at a time when necrotic lesions measured 12-80 mm. Fungicide absorption was

quite good only in the plants with restricted lesions; in one case the disease had encircled the stem, and this prevented any fungicide uptake. At 30 dpi, lesion length had reached 90-182 mm on the stem, and by 60 dpi, 30% of the treated plants had died. However, on the surviving plants, lesion length did not increase perceptibly (85-182 mm). Untreated control plants produced lesions of length 120-260 mm at 30 dpi, and 280-450 mm at 60 dpi. By 60 dpi, 80% of the plants had died (Fig. 1).

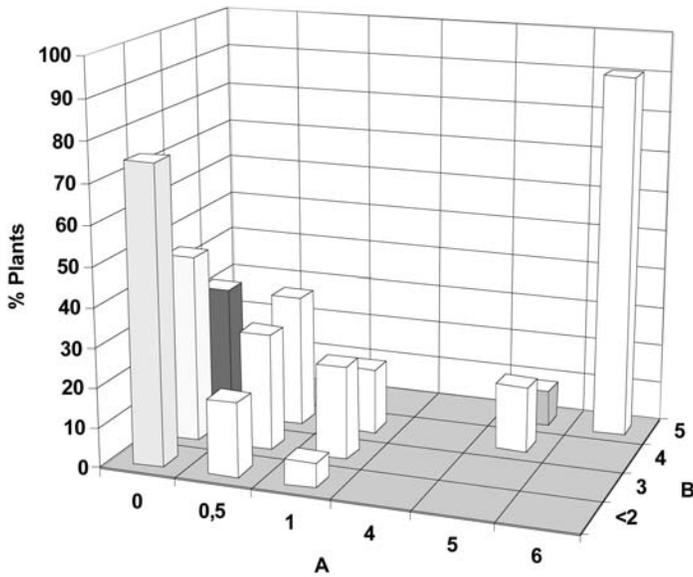


Fig. 2. The distribution of adult chestnut trees in classes of disease severity (A), after several applications of potassium phosphite, and (B), with respect to disease severity as measured by symptom expression at the time of the first treatment.

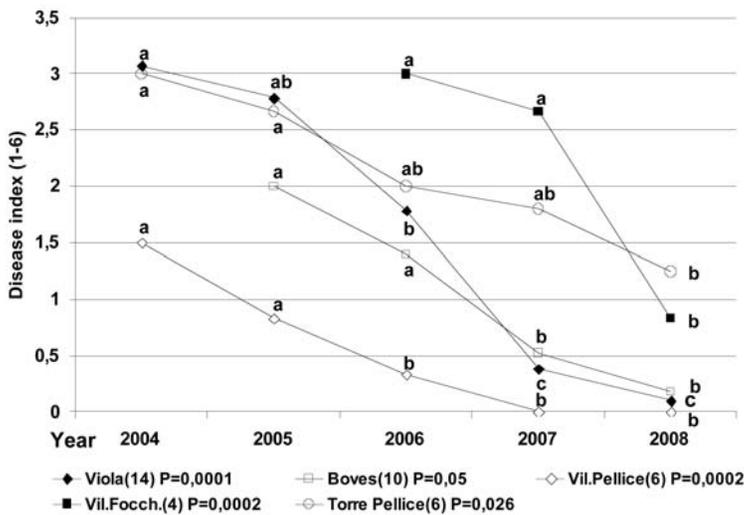


Fig. 3. Development of ink disease (*Phytophthora cambivora*) in adult chestnut trees growing at various sites in Piedmont, following an annual treatment with potassium phosphite. In brackets the number of treated plants at each site. P is the probability of error following LSD test relative to each site: means on the same line with the same letter are not statistically different.

Field trials. Ten of the 11 chestnut trees heavily colonised by *P. cambivora* (disease index 5) died within 1-2 years of the treatment, with the single survivor showing negligible symptom remission. Of the six trees having a disease index of 4, one died, but the others recovered, to the extent that by 2008, they were only rated 0-1 on the index scale. After 4-6 treatments, the trees showing a disease index of $\leq 1-3$ at the time of the first treatment were vegetatively vigorous, and were either symptomless or at worst, carried a few slightly undersized apical leaves (Fig. 2). Plant recovery was statistically significant from the time of the second year treatments, and behaved similarly at all sites (Fig. 3). The recovery of walnut was more rapid than in chestnut, reaching significance within a year of the first treatment in trees treated both in 2005 ($P= 0.0004$) and in 2006 ($P= 0.0002$), with a similar trend. Symptom severity in untreated trees increased dramatically and most trees had died within 3-4 years (Fig. 4).

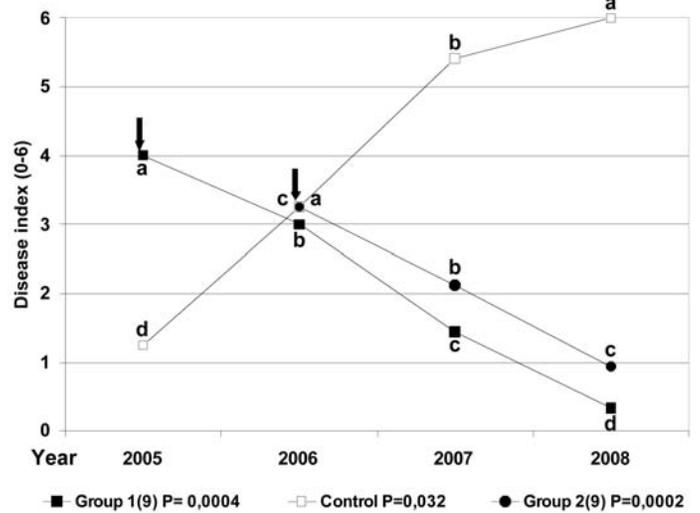


Fig. 4. Development of ink disease (*Phytophthora cinnamomi*) in walnut trees growing in Luserna S. Giovanni (Piedmont) following an annual treatment with potassium phosphite. Nine plants showing intermediate symptoms were treated in 2005, with 15 plants left untreated; in 2006, a second group of nine plants was randomly chosen among these latter 15 untreated trees. Arrows indicate the timing of the first treatment. In brackets the number of treated plants in each group. P is the probability of error following LSD test relative to each group: means on the same line with the same letter are not statistically different.



Fig. 5. Restriction of ink disease lesions in a chestnut tree treated with potassium phosphite.

Reduction in visual symptoms on the canopy was accompanied by production of woody tissue around the trunk lesions both in chestnut and walnut (Fig. 5). In chestnut there was some root growth, as shown by production of healthy sectors in the crown, and basal shoots of ever increasing number and vigour. Repair of drilling damage was rapid in healthy tissues but quite slow or incomplete in diseased tissue.

DISCUSSION

The Chiusa Pesio isolate of *P. cinnamomi* was selected after initial screening as the most virulent of the field isolates in our collection, and proved to be the most reliable for inducing disease on chestnut plants in the warm conditions of our nursery (data not reported). Since *P. cinnamomi* is also the causal agent of ink disease in walnut, it was used to provide inoculum in all the pot trials. One preventive, followed by three curative sprays of potassium phosphite together significantly reduced (by 87-90%) and then stopped the stem colonisation of young chestnuts artificially inoculated with *P. cinnamomi*. This result is in agreement with those ob-

tained with other phosphite derivatives (phosethyl-Al) against *P. cambivora* on chestnut nurseries (Skoudridakis and Bourbos, 1990). A single treatment by stem injection 12 days before inoculation was sufficient to fully inhibit the development of infection. The higher efficiency of injection (compared to foliar spraying) presumably reflects better access to host tissue, as well as the development of improved long-term fungicidal activity, as reported elsewhere (Diczbalis *et al.*, 2004). Potassium phosphite was also effective as a curative treatment, although both the ability of the tree to absorb the compound, and its fungicidal activity were affected by the level of disease severity at the time of treatment. Preliminary tests on potted plants revealed no toxicity, so the compound appears to be well suited for use on plants in the field.

Darvas *et al.* (1984) estimated the dosage of phosethyl-Al to be injected in fully grown avocado trees based on canopy area; we chose trunk diameter as our basis as this is much easier to measure. The MCE injection method has the advantage of needing only small diameter holes in the trunk, that are easy to be seal, and atmospheric pressure. Its main disadvantage is that absorption can be affected by factors such as the density and functionality of the canopy, the effectiveness of the plant vascular system, and all the climatic variables which can influence a plant's transpiration rate. Thus, treatment should preferably be given on sunny days, ensuring that absorption occurs correctly.

In all, 59 chestnut and 18 walnut trees were injected during field trials. Among the former, two very old trees in Torre Pellice (Turin) were felled by a storm, and 15 (in Scagnello and Robilante, Cuneo) were part of stands later incorporated into a system of organic production; thus these 17 trees could only be monitored over two years. At the time of the first treatment, the level of disease severity varied greatly from tree to tree. As observed in pot trials, treatment effectiveness (as measured by the number of symptomless trees after 3-5 applications) was inversely proportional to initial disease severity ($R^2 = 0.97$). As also observed for declining *Quercus ilex* and *Q. suber* (Fernandez-Escobar *et al.*, 1999), the benefit of treatment for chestnut only became evident after at least two years, while walnut responded within one year, perhaps because of the relatively young age of these trees.

Some recovering chestnut trees first produced large numbers of vigorous basal shoots, presumably because functionality of the root system had been restored. Both in chestnut and walnut, the improved plant health achieved by fungicidal treatment led to more vigorous sprouts, larger and more healthily coloured leaves, and more normal fruit development and ripening. These characters were all associated with the inhibition of ink flux, lesion dehydration and a disruption of the necrotic bark due to the swelling growth of wound regenerating

tissues. A similar compartmentalisation of disease lesions has been noted for *Banksia brownii* infected by *P. cinnamomi* following treatment with potassium phosphonate. Treatment in this case promoted the deposition of suberin and/or lignin both radially and tangentially in the xylem and longitudinally and tangentially in the bark, followed by regeneration of xylem, phloem, and periderm (Smith *et al.*, 1997).

Recently it has been shown that both *Xanthorrhoea australis* and *Arabidopsis thaliana* treated with potassium phosphite and inoculated with various *Phytophthora* spp. acquire a level of induced resistance (Daniel *et al.*, 2005; Daniel and Guest, 2005). Thus it is probable that, also in chestnut and walnut, potassium phosphite induces a number of defence reactions, which act to confine the pathogen by localised deposition of occlusive and protective compounds, followed by passive dehydration of infected tissue. Significantly, raising the tree water deficit proved sufficient to limit the colonisation and survival of *P. cinnamomi* in *Eucalyptus marginata* (Bunny *et al.*, 1995). It is also possible that potassium phosphite has a direct toxic effect on mycelial growth, sporulation, and zoospore germination (Raynal *et al.*, 1980; Smillie *et al.*, 1989; Reuveni, 1997), thereby helping to limit the inoculum load, and facilitating removal of the pathogens. This may be especially true for those with only poor ability to survive as saprophytes, as is the case for *P. cambivora* (Vettraino *et al.*, 2001). New infections were not observed following treatment of the chestnut trees.

From a practical point of view, the proposed method is simple and cheap to use, and allows for the direct delivery of a proportionate quantity of fungicide to the plant. It does not appear to induce any negative side-effects, and drillings made in healthy wood were all sealed within the season. Fungicide sprays were also effective, and so are recommended for use in chestnut and walnut nurseries to prevent infections by *Phytophthora* spp.

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