Phytopathol. Mediterr. (2009) 48, 379–384

RESEARCH PAPERS

Effect of bioagents and resistance inducers on grapevine crown gall

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Summary. Bioagents and chemicals were applied to one-year old grafted vines (Ancelotta/420A) in glasshouse and field experiments set up at the Vivai Cooperativi Rauscedo (VCR, Pordenone, Italy). In the glasshouse, holes were drilled in vines on the rootstock and the holes were charged with suspensions of different strains of *Pseudomonas* spp., and with the biofungicides BS-F4 and Serenade, both based on *Bacillus subtilis*, before inoculation with a vitopine *Agrobacterium vitis* strain. The growth retardant Regalis and the resistance inducer Bion were applied to the vines two weeks before inoculation with the pathogen. Six months after inoculation, disease incidence was lowest when BS-F4 had been applied. In the field trial, the vines were wounded by making a cut in the crown, after which they were dipped into the antagonist suspensions just before inoculation with the pathogen. In the two weeks before inoculation, the root systems of the vines were dipped into Regalis and Bion solutions at 7 day intervals. Only these resistance inducers and BS-F4 significantly reduced disease severity. The results indicate that a potential for defence against *A. vitis* may exist even in susceptible grapevine cultivars, and that this potential can be activated by diverse elicitors.

Key words: *Agrobacterium vitis*, tumour, resistance inducers, antagonists.

Introduction

The systemic survival of *Agrobacterium vitis* in grapevines and the ease with which this pathogen spreads within the host makes it difficult to control crown gall, thus making it essential to adopt preventive measures on the plant and the agrobacteria. A number of practical guidelines should be followed in the planning phase of vineyard establishment, in viticultural methods, as well as in indexing and certifying propagation materials (Burr *et al*., 1998; Burr and Otten, 1999). It is important to detect and identify *A. vitis* in nursery vines and soils, and the high genetic diversity of agrobacteria may require the use of multiplex PCR techniques to do so (Bini *et al*., 2008b). Unfortunately, no chemical controls are available even though copper compounds (e.g.: bordeaux mixture) and pre-graft oxyquinoline applications may have some efficacy after leaf fall and at the end of the dormant period. Another control strategy is the use, whenever possible, of resistant rootstocks and scion cultivars reducing pathogen survival and inhibiting oncogene expression (gene silencing) (Burr *et al*., 2003; Kovács *et al*., 2003). T.J. Burr has stated that, "taking the fight at the genetic level may provide a possible long-term protection and an alternative strategy for an effective and sustainable control of the disease" (Burr *et al*., 2003). Shoot-tip propagated vines in soils where grapevines had not previously been grown and thermotherapy of dormant cuttings are other available means of control; hot water treatments (Bazzi *et al*., 1991; Waite and Morton, 2007) significantly reduce pathogen populations in fully dormant cuttings used for propagation, and heat-shock treatments with hot air are helpful in improving the thermotolerance of propagation material. Particular attention should also be

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given to biological control, with the selection and use of antagonist micro-organisms that have a direct or an indirect action (induced resistance). Various *Agrobacterium* and *Pseudomonas* spp. strains have been used as biocontrol candidates (Staphorst *et al*., 1985; Bell *et al*., 1995; Khmel *et al*., 1998; Kawaguchi *et al*., 2007).

The aim of this study was to evaluate the effectiveness of various strains of *Pseudomonas* spp. and two chemical resistance inducers in reducing crown gall incidence and severity under glasshouse and field conditions.

Materials and methods

Glasshouse experiment

Grapevines cv. Ancellotta (*Vitis vinifera*) grafted on 420A rootstock (*V. berlandieri* × *V. riparia*) evenly grown in pots, were used in the experiment (5 vines × 5 replicates per treatment distributed in randomized blocks). Three holes, 4 mm in diameter and 8–10 cm apart, were drilled into each rootstock to the depth of the pith (a total of 75 wounds per treatment) and charged with 60 µL suspensions (approx. 10^8 cfu mL^{-1}) (Burr and Reid, 1993) of the *Pseudomonas* spp. strains IPV-BO G19, IPV-BO 4027C (Bazzi *et al*., 2006a; Bazzi *et al*., 2006b), CR330d and Pf 1 (Khmel *et al*., 1998), and with suspensions of the biofungicides BS-F4 (Alexandrova *et al*., 2002; Bazzi *et al*., 2006a) and Serenade (Edgecomb *et al*., 2006), both based on *B. subtilis*. After about 30 min, a suspension (approx. 10^8 cfu mL^{-1}) of *A. vitis* from cells of the vitopine strain IPV-BO 5159 grown on YMA ($MgSO_4$×$7H_2O$ 0.2 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, Mannitol 10 g L^{-1}, yeast extract 0.4 g L^{-1}, NaCl 0.1 g L^{-1}, Bacto Agar 15 g L^{-1}; pH 7.2) for 48 h at 27°C was inoculated into the holes and the inoculation sites were wrapped with Parafilm. In the two weeks before pathogen inoculation, the growth retardant Regalis (10% WG prohexadione-Ca, BASF, Ludwigshafen, Germany) and Bion (50 WG acibenzolar-S-methyl, Syngenta, Basel, Switzerland) were sprayed on the shoots three times at 7-day intervals (at 1 × 75 ppm, 1 × 50 ppm and 2 × 100 ppm respectively). Copper oxychloride (2 g Cu^{++} L^{-1}, standard negative control) and water (positive control), as well as the antagonist suspensions, were applied before inoculation with *A. vitis*. Disease incidence (DI: % of galled wounds) and disease severity (DS: mean tumour diameter, mm) were determined 6 months later (Figure 1) and the data were statistically analysed (LSD, P=0.05) using SPSS 15.0 for Windows® and StatGraphic 2.1. The pathogen was reisolated on the semi-selective RS medium (per L: $MgSO_4$ • $7H_2O$ 0.2 g, K_2HPO_4 0.9 g, KH_2PO_4 0.7 g, adonitol 4 g, yeast extract 0.14 g, NaCl 0.2 g, H_3BO_3 1 g, Chlorothalonil [Bravo 500] 4.0% [w:v] 0.5 mL, Bacto Agar 15 g; pH 7.2; to which was added aseptically Trimethoprim 20 mg, Triphenyltetrazolium chloride 80 mg and D-cycloserine 20 mg) (Roy and Sasser, 1983; Moore *et al*., 2001) from 100 mg of fresh tumour tissue collected at the wound sites. Bacterial colonies were identified by PCR assay (Haas *et al*., 1995). Agronomical parameters such as shoot and root length, root fresh weight and rootstock diameter were used to evaluate the effect of the treatments on vine growth.

Field experiment

The vines cv. Ancellotta/420A (5 vines × 5 replicates per treatment, distributed in randomized blocks) were cut at the crown and dipped into the antagonist suspensions IPV-BO G19, IPV-BO 4027C, CR330d and Pf 1–3 and the biofungicide suspensions BS-F4 and Serenade (same concentrations as those used in the glasshouse experiment), into the copper oxychloride suspension (2 g Cu^{++} L^{-1}, standard negative control) and into water (positive control). After approx. 15 min, the vines were further dipped for 10 min into an *A. vitis* suspension (approx. 10^8 cfu mL^{-1}) and were then transplanted to the vineyard (Bazzi *et al*., 1998). In the two weeks before inoculation, the root systems of the vines were dipped into Regalis and Bion solutions at 7-day intervals, as in the glasshouse experiment. DI (% of galled vines) and DS were determined 8 months after inoculation (Figure 1) and the data were statistically analysed (LSD, P 0.05) using SPSS 15.0 for Windows® and StatGraphic 2.1. The pathogen was reisolated on RS medium from 100 mg of tumour tissue and bacterial colonies were identified by PCR assay. As in the glasshouse experiment, vine growth parameters were evaluated for each of the treatments.

Results

In the glasshouse, the effect of BS-F4, IPV-BO G19, Regalis and Bion did not differ from that of copper oxychloride. BS-F4 most significantly reduced the percentage of galled wounds (32.0%) and DS, limiting mean tumour diameter to 4.3 mm, vs. 12.9 mm in the positive control. The standard negative control copper oxychloride significantly reduced both DI (46.7% of galled wounds) and DS (mean tumour diameter 6.3 mm) (Table 1).

In the field, none of the treatments significantly reduced DI as compared with the water control, although Regalis gave the apparent lowest level of symptomatic grapevines (68.0%). Only Bion and Regalis significantly reduced DS, with a mean tumour diameter of 12.9 and 16.6 mm respectively, compared with 30.1 mm for the positive control.

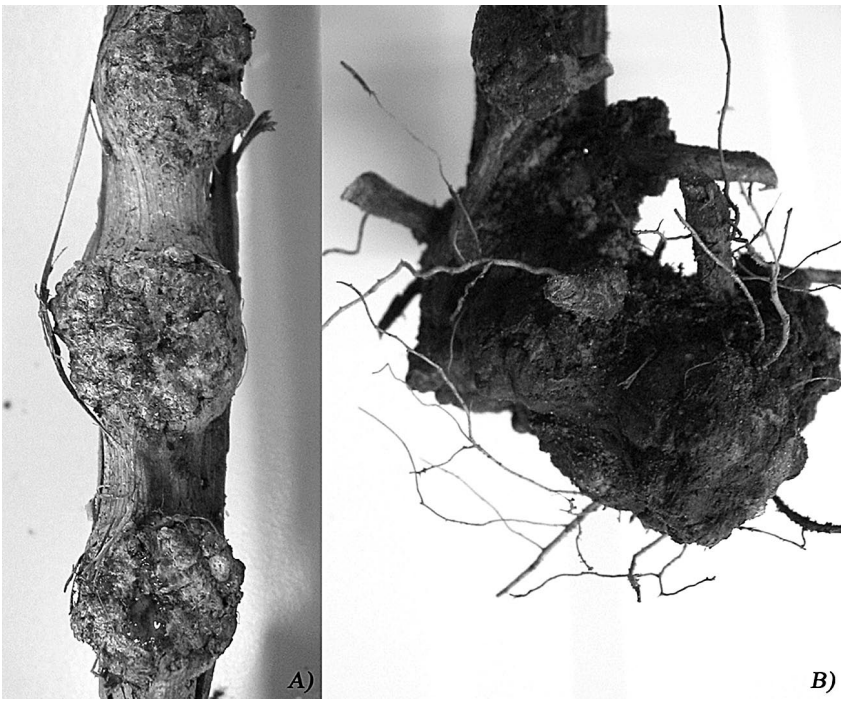

Figure 1. A) Tumours in the '420A' rootstock of a grafted vine inoculated with *Agrobacterium vitis* in the glasshouse (positive control); B) Evident neoplastic formation at the crown of a grafted vine grown in the field 8 months after inoculation with *A. vitis* (positive control).

Table 1. Glasshouse experiment. Disease incidence (DI) related to the percentage of tumoured sites experimentally inoculated with *Agrobacterium vitis*. Disease severity (DS) was evaluated on the basis of mean tumour diameter.

Treatment	DI, Galled wounds (%)	DS, Mean tumour diam. (mm)
Water (positive control)	82.7 c[a]	12.9 c
CR330d	65.3 bc	9.9 bc
Serenade	65.3 bc	9.2 abc
Pf 1-3	57.3 abc	7.3 ab
IPV-BO[b] 4027C	56.0 abc	5.9 ab
Regalis	50.7 ab	5.5 ab
Copper oxychloride (negative control)	46.7 ab	6.3 ab
Bion	45.3 ab	4.8 ab
IPV-BO G19	42.7 ab	4.8 ab
BS-F4	32.0 a	4.3 a

[a]Different letters within columns denote significant differences according to the LSD test (P=0.05).
[b]IPV-BO = Istituto Patologia Vegetale, University of Bologna, Bologna, Italy.

Table 2. Field experiment. Disease incidence (DI, percentage of grapevines showing tumours) and disease severity (DS, mean tumour diameter).

Treatment	DI, Galled vines (%)	DS, Mean tumour diam. (mm)
Water (positive control)	88.0 a[a]	30.1 cd
Serenade	88.0 a	26.2 abcd
IPV-BO[b] 4027C	84.0 a	21.8 abcd
Copper oxychloride (negative control)	80.0 a	34.8 d
Pf 1-3	78.0 a	26.5 bcd
CR330d	78.0 a	25.2 abcd
Bion	74.0 a	12.9 a
BS-F4	72.0 a	18.0 abc
IPV-BO G19	72.0 a	21.6 abcd
Regalis	68.0 a	16.6 ab

[a], [b] See Table 1.

Copper oxychloride did not differ from the water control in reducing DI or DS (Table 2).

Neither in the greenhouse nor in the field was the vegetative growth (shoot length, root length and weight, rootstock diameter) of the grapevines significantly affected by either the *Pseudomonas* spp. strains or Regalis (Rademacher and Kober, 2003).

Discussion

Treatments in the glasshouse in some cases, produced results differing from those in the field, possibly due to different experimental protocols. Specifically, the effectiveness that BS-F4, IPV-BO G19, IPV-BO 4027C and Serenade had shown in previous studies against fire blight (*Erwinia amylovora*) on apple and pear shoots and flowers, when they exhibited good colonisation, survival and protective ability (Bazzi *et al*., 2006a, b; Biondi *et al*., 2006) was not duplicated in grapevine by reducing crown gall incidence in the field. But the soil niche, is a very complex habitat, involving both biotic and abiotic factors, in which it is difficult for any newly introduced bacterial inoculum to act as an antagonist against *A. vitis* and also to live and persist in the vineyard soil (Burr *et al*., 1995).

Another important aspect is the lack of activity shown in the field by the standard negative control, copper oxychloride, chosen in place of the antibiotic streptomycin whose use in Italy has been banned since 1971. In general, the prohibition of antibiotics and limitations placed on copper compounds has promoted a search for alternative means to protect crops and to ensure a sustainable agriculture. Our study, represented an additional attempt in this direction, following earlier studies carried out in Italy by Bazzi *et al*. (1998) in which various pseudomonads and *B. subtilis* preparations were tested for the biological control of grapevine crown gall. In nature, pseudomonads down-regulate the growth of agrobacteria (Eastwell *et al*., 2006), but their real modes of action and their efficacy under different conditions remain to be investigated. Galasso *et al*. (2002) reported that the *Pseudomonas fluorescens* strain IPV-BO G19 produced *in vitro* a polypeptidic antibacterial compound, but other possible modes of biocontrol action by the pseudomonads such as the production of the polyketides 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine-1-carboxylic acid (PCA), pyoluteorin (Plt) and pyrrolnitrin (Prn) (Raaijmakers *et al*., 1997; Nowak-Thompson *et al*., 1999) are still being investigated. So far, the non-pathogenic *A. vitis* F2/5 from South Africa (Staphorst *et al*., 1985) seems to be the most promising in preventing crown gall on grapevines, and recent studies suggest that the agrocin-independent biocontrol activity of such a strain is caused by necrotic reactions induced on the grapevine cambium (Creasap *et al*., 2005). F2/5 has a complex quorum-sensing regulatory system that is associated with a hypersensitive response on tobacco and with a necrotic reaction on grapevine (Hao *et al*., 2005). However, the wild F2/5 strain is not effective against all tumorigenic strains, and plants treated with F2/5 may exhibit tissue necrosis, graft incompatibility and mortality.

These problems represent important limits to the practical use of F2/5 on a commercial scale. An efficient biocontrol agent should not only be antagonistic and survive stably in the target niche, but should also be devoid of any detrimental effect *in planta*.

The resistance elicitors Regalis and Bion both had a significant effect on DI and DS under glasshouse and field conditions. Even if Regalis is not one of the conventional phytosanitary products on grapevine, and only preliminary tests of this elicitor against *Plasmopara viticola* have been done (Bazzi *et al*., 2003), it will be important to study at molecular level the inducible defence mechanisms evoked by these intriguing signal molecules. Such inducer molecules are linked to an increased expression of a number of pathogenesis-related genes (*pr* genes) leading to an accumulation of pathogenesis-related proteins (*pr*s) in plant tissues, markers of systemic acquired resistance (SAR) and/or induced systemic resistance (ISR) (Hamiduzzaman *et al*., 2005). The transcriptional activity of the genes of some important PRs increased in self-rooted apple trees after Regalis was applied (Bini *et al*., 2008a). Another beneficial side effect of this dioxygenase inhibitor is that it brings about crucial changes in the metabolic pathways of flavonoids and phenylpropanoids in plant tissues, increasing resistance against various diseases (Spinelli *et al*., 2006). It would therefore be interesting to see if these unique compounds (e.g.: luteoforol) also occur in grapevines treated with Regalis.

Further research is focusing on how biocontrol can prevent crown gall, and how important it is to reach a threshold population that will offer persistent protection in different agroecosystems. The role of the highly structured and persistent microbial bio-films that form in wounds is also being investigated. It will also be essential to determine the most effective means to apply the beneficial organisms (a root dip before planting creating a lifelong shield, a vine injection, or an annual spray?). Other biotic and abiotic molecules that are potential elicitors of resistance responses will also be explored. Answers to these and other questions are necessary to develop a preventive strategy against this neoplastic grapevine disease.

Acknowledgements

This work was supported by the grapevine nurseries Vivai Cooperativi Rauscedo (VCR, Pordenone, Italy). We are grateful to Prof. Thomas J. Burr (NYSAES, Cornell University, Geneva, NY, USA) for careful reading of the paper, and to Prof. Maria C. Sanguineti (DiSTA, University of Bologna) for assistance with the statistics.

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Accepted for publication: July 27, 2009