Vitis **38** (1), 31–35 (1999)

Biological control of *Agrobacterium vitis* using non-tumorigenic agrobacteria

by

C. Bazzi[1], M. Alexandrova[1], E. Stefani[1], F. Anaclerio[2] and T. J. Burr[3]

[1] U.C.I., Scienze e Tecnologie Agroindustriali e Agroambientali, Istituto di Patologia Vegetale, Bologna, Italia
[2] Vivai Cooperativi di Rauscedo, Pordenone, Italia
[3] Department of Plant Pathology, New York State Agricultural Experiment Station, Geneva, USA

S u m m a r y : The potential use of non-tumorigenic agrobacteria for the biological control of grapevine crown gall in Italy was investigated. Four *Agrobacterium* strains belonging to the species *radiobacter* and *vitis* were used to protect the susceptible cv. Malvasia Istriana grafted on the rootstock 420 A. Moreover, the effect of each treatment on grapevine vitality and growth was assessed, including the percentage of marketable vines, as determined by industry standards. Treatments with the antagonists clearly reduced tissue colonization by the pathogen, with a drop of more than 100-fold in pathogen populations in the samples collected at the graft point. Another important effect was the reduction of internal necrosis possibly induced by the high concentration of the nopaline strain CG 49 used in the experiments. According to viticultural and commercial parameters, treatments with the antagonists improved the quality of the vines, with fewer discards and a high percentage of marketable material. Therefore, these antagonists can be considered beneficial for grapevine.

K e y w o r d s : grapevine, crown gall, Agrobacterium vitis, biological control, antagonists.

Introduction

The systemic survival of *Agrobacterium vitis* in grapevine and the ease with which the pathogen can spread within the host (Lehoczky 1968) makes it difficult to control crown gall, thus making essential to adopt preventive measures on the plant and the agrobacteria. Control is based on viticultural criteria (Boubals 1987; Süle *et al.* 1994, 1995; Stover *et al.* 1997) as well as on the indexing and certification of propagation material (Kauffman *et al.* 1996; Szegedi and Nemeth 1996). Chemical treatments can be made with copper compounds or with oxyquinoline sulphate (Faivre-Amiot 1984; Cazelles *et al.* 1991), but their efficacy, although occasionally significant, is not satisfactory. Another control measure used in the nursery consists of hot water treatment to eradicate *A. vitis* from dormant grape cuttings (Bazzi *et al.* 1991; Burr *et al.* 1996).

Recently, particular attention was given to biological control with the selection and use of antagonist microorganisms (Staphorst *et* al. 1985; Webster *et al.* 1986; Xiaoying and Wangnian 1986; You *et al.* 1990; Pu and Goodman 1993; Burr and Reid 1994), which can have a direct (antibiosis, competition for target sites or nutrients) or indirect action (induced resistance in the host).

The main purpose of this study was to assess the practical potential of biological control of grapevine crown gall using non-tumorigenic agrobacteria in Italian viticulture.

Materials and Methods

B a c t e r i a l s t r a i n s : The authentic strains of *Agrobacterium* spp. used in this study are listed in Tab. 1 together with their geographic origin. The tumorigenic strain CG 49 of *A. vitis* (having a nopaline type Ti plasmid) was used as a positive control. The pure bacterial cultures were stored at -80 °C in NUNC Cryotubes containing LB broth (Bertani 1952) with 15% glycerol. The bacterial cultures were routinely grown on YMA (Miller *et al.* 1990).

G r a p e v i n e m a t e r i a l : The susceptible cv. Malvasia Istriana (*Vitis vinifera*) and the rootstock 420 A (*V. berlandieri* x *V. riparia*) were used. Canes of various lengths were collected from asymptomatic dormant mother vines in the Rauscedo nurseries (Pordenone). Samples were indexed for *A. vitis* using the method of Bazzi *et al.* (1987) together with the monoclonal antibody of Bishop *et al.* (1989) in dot-immunobinding assays (DIA).

B a c t e r i a l i n o c u l u m : The bacteria were grown on YMA for 48 h at 28 °C, suspended in sterile distilled water (SDW) at an absorbance of A_{660} = 0.5, containing between 6.6×10^8 and 2×10^9 CFU·ml^{-1}.

T r e a t m e n t w i t h a g r o b a c t e r i a : Cuttings (length: 30 and 40 cm) were taken from the rootstock and scion cultivar, the latter with an average of 4 nodes. The experimental design consisted of 6 different treatments with 4 replications of 20 cuttings each. Individual bunches of

Research supported by Grant no. 95.02483.06 from CNR, Rome, Italy, under Italy-USA bilateral research project "Grapevine crown gall: Significance of *Agrobacterium vitis* from native vines and studies on biological control mechanisms" and N.Y. Wine and Grape Foundation.

Correspondence to: Prof. Carlo Bazzi, Istituto di Patologia Vegetale, Via Filippo Re 8, I-40126 Bologna, Italy. Fax: +39-51-351446. E-mail: cbazzi@pop.agrsci.unibo.it

Table 1

Agrobacterium spp. strains used in the experiments

Species	Strain	Origin	References
A. radiobacter bv. 1	HLB-2	Hop, P.R. China	XIAOYING 1986
A. vitis	F2/5	Grapevine, South Africa	STAPHORST *et al.* 1985
A. vitis	CG 1077	Agrocin$^{(-)}$ mutant of F2/5, USA	BURR *et al.* 1997
A. vitis	CG523	*V. riparia*, USA	BURR and REID (unpublished)
A. vitis	CG49	Grapevine, USA	OTTEN *et al.* 1996

20 cuttings were inserted, first with their basal ends and subsequently with their apical ends in a special glass cylinder and preinfiltrated under vacuum (5 min, 100 kPa) with suspensions of the antagonistic *Agrobacterium* strains. The negative control was preinfiltrated with SDW. After 24 h, the cuttings preinfiltrated with the 4 antagonists (strains F2/5, CG 1077, CG 523 and HLB-2) were infiltrated with a suspension of the virulent strain CG 49; the positive control (not preinfiltrated) was infiltrated with the same suspension of the pathogen. After treatment, all cuttings were kept for 48 h at room temperature, then transferred to a cold room (2 °C) until grafting. Bench grafting and forcing was performed following the standard nursery techniques (BAZZI *et al.* 1991). For each treatment 20 grafted vines were transplanted into pots and grown in the greenhouse and 60 were transplanted to a field near Cordenons (Pordenone) using a single-row block randomised design.

Protective action of the antagonist: The action of the antagonists on the tumorigenic agrobacteria populations was assessed 8 months after transplanting. In the greenhouse, the number of surviving vines was counted for each treatment as well as the presence of tissue necrosis and the number of vines with tumours.

For the vines in the nursery, the direct action of the biological controls on the CG 49 populations was determined by collecting pieces of tissue (150 - 500 mg depending on availability) close to the graft-points for quantitative isolation on semi-selective RS medium (ROY and SASSER 1983). When all the vines were dug up, the percentage of surviving vines was determined for each treatment, as well as the presence of external and internal tissue necrosis and the development of tumours.

Identification of *A. vitis* (strain CG 49): When collection of tissue was possible, out of each treatment, three representative colonies/plate/vine were selected from the isolation plates and purified; then the bacterial isolates were grown overnight on LB broth at 27 °C in a rotary shaker. From 20 plants of each treatment the following number of tissue samples were assayed: HLB2: 11/20; 523: 5/20; CG 1077: 1/20; CG 49: 7/20; F2/5: 0/20. Bacterial suspensions were centrifuged and the pellets resuspended in SDW, absorbance of $A_{660} = 0.1$ (ca. 10^8 CFU·ml^{-1}) and subjected to RAPD analysis. The same analysis was performed on the genomic DNA solutions extracted from the same bacteria. DNA extraction was performed according to WILSON (1989). All amplification reactions were performed with the thermocycler GenAmp PCR System 2400 (Perkin Elmer) using the primer 10-mer 60-30 (5'GAGCAGGCTG3') (BURR *et al.* 1995). The amplicons were separated during electrophoretic runs (SAMBROOK *et al.* 1989).

Effects on vine growth: The assessment was made 8 months after transplanting the vines in the nursery. The following parameters were calculated: percentage of graft-take on the total number of surviving vines, wood and root production (g), marketable vines (vines that can be sold commercially as determined by industrial standards), dead and discarded vines. The data were analysed by analysis of variance using a complete randomised block design and the mean values of the treatments were compared applying the mean significant difference (MSD). Growth of the vines in the greenhouse was assessed.

Results

The analyses performed on plant material, collected from asymptomatic mother vines, were negative: 28 bacterial strains morphologically similar to *A. vitis* isolated from this material did not react with the species-specific monoclonal antibody used in the dot-immunobinding assay (DIA).

Two months after transplant in the greenhouse and in the nursery, a progressive and extensive necrosis was observed on the vascular parenchyma, at graft and throughout the vines. Eight months after transplant, direct isolations on RS medium and identification of the selected colonies with arbitrary amplification polymorphism analysis (RAPD) of total genomic DNA made it possible to assess the presence of 1.24×10^6 to 7.35×10^6 CG 49·g^{-1} of tissue from the vines treated with the antagonists and 2.7×10^8 bacteria·g^{-1} of tissue from the positive controls; no bacteria were isolated from the negative controls (Tab. 2).

The vitality and quality of the vines in the nursery are summarised in Tab. 3. The surviving vines were 48.8 and 89.0 % for the positive and negative controls, respectively. In general, the treatments with the antagonists revealed a reduction in colonization by CG 49. The highest vitality was shown by the vines treated with the agrocin-minus mutant of F2/5, CG 1077, with a percentage of surviving vines of 83.8 %, not significantly different from the negative control. In the greenhouse experiment the virulent strain CG 49 caused a high mortality of the vines (17/20, the surviving three had tumour masses) while those treated with the antagonists showed an effective protection. In particular, the number of dead vines was 4/20 for the treatment with F2/5, 2/20 with

Table 2

Effect of the antagonists on the colonization of grapevine tissue by *Agrobacterium vitis*

Contaminating dose dose CFU·ml^{-1} (strain CG 49)	Treatment	Number of bacteria per g fresh weight (strain CG 49)
10^8 - 10^9	HLB-2	7.35×10^6
" "	F2/5	N.D.*
" "	CG1077	1.24×10^6
" "	CG523	1.26×10^6
" "	positive control	2.70×10^8
H_2O	negative control	0

* no samples collected due to excessive lignification of tissue at graft point.

CG 1077, 13/21 with CG 523 and 9/20 with HLB-2; in the latter case there were also two vines with tumours, while tumours were not observed on the other surviving vines treated with the biocontrols.

In the nursery and in the greenhouse, death of the grafted vines treated with the pathogen was preceded by poor growth and collapse. Extensive necrosis of the woody parenchyma were particularly obvious at the transverse and longitudinal cross-sections. There were very few hyperplasias which could be attributed to tumours, especially on the vines in the nursery. In the greenhouse only 3/20 and 2/20 vines showed tumours, amongst those treated with the strains CG 49 (positive control) and CG 523.

As regards the vitality and growth of the treated vines, graft-take ranged from 83.9 to 100 %; only the vines treated with F2/5 showed a significantly lower value (67.5 %). There was no significant difference in wood or mean root production for the various treatments, except for the vines treated with strain CG 523, which had a slight rhizogenic effect.

Strains HLB-2, CG 523 and CG 1077 gave 69.4, 63.0 and 60.3 % marketable vines, respectively, as compared with 32.9 % vines infiltrated with CG 49 alone. There was a very high percentage (67.1 %) of discarded vines with the positive control as compared with the negative one (17.8 %). Interestingly, the vines infiltrated with F2/5 had a high mortality (63.2 %), while the other treatments showed similar mortality rates, halfway between those of the positive and the negative controls.

Discussion

The efficacy of treatment with the antagonists was demonstrated by the reduction in pathogen populations *in planta* as well as in viticultural and commercial terms. In the first case, there was a lower colonization of grapevine tissues by *A. vitis* CG 49 infiltrated 24 h after the non-tumorigenic biological control candidates. The drop of more than 100-fold in the pathogen populations in tissue samples collected at the graft point might be the result of competition for nutrients and/or the direct action of antibacterial substances produced by the antagonists. PU and GOODMAN (1993) presented experimental evidence on the excellent ability of the strain HLB-2 to multiply in the host tissue and suppress tumour formation by competing for competent infection sites and nutrients and by producing an agrocin-like substance. Similarly, the avirulent strain of *A. vitis* F2/5 (STAPHORST *et al.* 1985) is confirmed to be an antagonist in the United States (BURR and REID 1994), inhibiting the growth of a high number of tumorigenic strains of different geographical origins and only some isolates of *A. tumefaciens* biovar 1 and 2 were insensitive to its action. It appears from

Table 3

Control of grapevine crown gall with antagonistic agrobacteria: nursery experiments

Antagonist	Vitality[1]	Graft-take[2]	Wood (g)	Roots (g)	Grafted vines I category[3]	Grafted vines II category[4]	Grafted vines I + II	Dead + discarded
F2/5	61.8 c	67.5 b	17.33	18.54 ab	74.2 b	17.5 a	36.8 c	63.2 a
CG 1077	83.8 a	83.9 ab	19.19	17.56 b	69.8 b	16.8 a	60.3 b	39.7 b
CG 523	67.6 bc	100 a	18.79	21.01 a	91.0 a	2.5 c	63.0 b	37.0 b
HLB-2	72.2 b	100 a	19.66	14.05 c	89.3 a	7.2 abc	69.4 b	30.5 b
contr. +	48.8 d	97.5 a	20.40	18.25 ab	63.5 b	6.9 bc	32.9 c	67.1 a
contr. -	89.0 a	100 a	18.14	15.82 bc	77.2 b	15.3 ab	82.2 a	17.8 c
Test F	**	*	n.s.	**	**	*	**	**

The means with different letters are significantly different (P≤0.05) (Test DMS).
Significance of F test at a probability level of 0.01 (**), 0.05 % (*) and not significant (n.s.).
[1] Surviving plants out of total plants.
[2] Graft-take on surviving plants.
[3] Percentage calculated on graft-take.
[4] Percentage calculated on total plants.

our work that agrocin production by F2/5 is probably not an important factor related to its ability to reduce CG 49 populations in grape. We showed that strain CG 1077 (an agrocin-minus mutant of F2/5) was effective in reducing CG 49 populations and previously it was shown that CG 1077 and other agrocin-minus derivatives of F2/5 were as effective as the wild-type strain for preventing gall formation on grape (BURR *et al.* 1997).

The results of our experiments indicated that high concentrations (ca. 10^8 CFU·ml^{-1}) of the nopaline strain CG 49, vacuum-infiltrated in cuttings was highly necrogenic on the xylem-parenchyma tissue of the grafted vines. The response was poor vine growth, collapse and mortality rather than tumorigenicity. Therefore necrogenesis seems to drastically limit the success of tumour transformation as observed by PU and GOODMAN (1992) on grapevines and on explants inoculated with an aqueous suspension ($5x10^6$ per stem piece) of the supervirulent strain A 281; in this case, 84 % of the cv. Chancellor explants developed necrosis. Further investigation is necessary for a better understanding of the cause of necrosis induced by *A. vitis* under some circumstances and to define the controlling factors. Some hypotheses can be made that are merely speculative: the lethal effect of excessive levels of accumulated auxin on plant cells; the pectolytic activity of strain CG 49 which has the chromosomal gene *pehA*, coding a single polygalacturonase that degrades plant cell walls (BURR *et al.* 1987).

On the basis of viticultural and commercial parameters, it can be said that treatment with the antagonists had a clearly stimulating effect combined with fewer discards and a high percentage of marketable vines. For this reason, these bacteria can be considered as beneficial for grapevine, like strain K-84, which can stimulate the growth of apple seedlings (BAZZI 1981), and other organisms (*Azotobacter*, *Bacillus*, *Pseudomonas*, *Clostridium* spp.) known as PGPR (BURR and CAESAR 1984) used for the bacterisation of seeds, tubers and roots. The large-scale use of antagonistic agrobacteria might be useful for the control of the grapevine replant syndrome where *A. vitis* might be one of the numerous factors involved (BAZZI and BURR 1987). More detailed studies are necessary to examine the action mechanisms of these non-tumorigenic agrobacteria and their ability to exclude the pathogen, predominating in the colonization of grapevine tissues during microbial interaction in different agroecosystems, so as to develop and optimise its application on a large scale as a protective measure.

Acknowledgement

The authors would like to acknowledge Vivai Cooperativi di Rauscedo, Pordenone, Italy for technical support.

References

BAZZI, C.; 1981: Biological control of plant pathogens. Bulletin OEPP/EPPO **11**, 119-126.

- -; BURR, T. J.; 1987: Le malattie da reimpianto della vite e dei fruttiferi. Inform. Fitopatol. **12**, 23-25.

- -; PIAZZA, C.; BURR, T. J.; 1987: Detection of *Agrobacterium tumefaciens* in grapevine cuttings. Bulletin OEPP/EPPO **17**, 105-112.

- -; STEFANI, E.; GOZZI, R.; BURR, T. J.; MOORE, C. L.; ANACLERIO, F.; 1991: Hot water treatment of dormant grape cuttings: Its effects on *Agrobacterium tumefaciens* and on grafting and growth of vine. Vitis **30**, 177-187.

BERTANI, G.; 1952: Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**, 293-300.

BISHOP, A. L.; MITTAK, V. L.; KATZ, B. H.; BURR, T. J.; 1989: A monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3 and its utilization for indexing grapevine propagation material. Phytopathology **79**, 995-998.

BOUBALS, D.; 1987: Conduite à tenir dans les vignes presentant des broussins. Prog. Agric. Vitic. **104**, 367-368.

BURR, T. J.; BISHOP, A. L.; KATZ, B. H.; BLANCHART, L. M.; BAZZI, C.; 1987: A root specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. Phytopathology **77**, 1424-1427.

- -; CAESAR, A.; 1984: Beneficial plant bacteria. In: CRC Critical Reviews in Plant Science **2**, 1-20.

- -; REID, C. L.; 1994: Biological control of grape crown gall with non-tumorigenic *Agrobacterium vitis* strain F2/5. Amer. J. Enol. Viticult. **45**, 213-219.

- -; - -; SPITTSTOESSER, D. F.; YOSHIMURA, M.; 1996: Effect of heat treatments on grape bud mortality and survival of *Agrobacterium vitis in vitro* and in dormant grape cuttings. Amer. J. Enol. Viticult. **47**, 119-123.

- -; - -; TAGLIATI, E.; BAZZI, C.; SÜLE, S.; 1997: Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. Phytopathology **87**, 706-711.

- -; - -; YOSHIMURA, M.; MOMOL, E. A.; BAZZI, C.; 1995: Survival and tumorigenicity of *Agrobacterium vitis* in living and decaying grape roots and canes in soil. Plant Dis. **79**, 677-682.

CAZELLES, O.; ESPARD, S.; SIMON, J. L.; 1991: Influence de la desinfection au sulfate d'oxyquinoleine du portgreffe Berl. x Rip. 5C sur l'expression du broussin lors de la multiplication de la vigne. Rev. Suisse Viticult. Arboricult. Hort. **23**, 285-288.

FAIVRE-AMIOT, A.; 1984: Les tumeurs à *Agrobacterium*. Phytoma **362**, 27-31.

KAUFFMAN, M.; KASSEMEYER, H.-H.; OTTEN, L; 1996: Isolation of *Agrobacterium vitis* from grapevine propagating material by means of PCR after immunocapture cultivation. Vitis **35**, 151-153.

LEHOCZKY, J.; 1968: Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine after natural infection. Phytopathol. Z. **63**, 239-246.

MILLER, K. J.; GORE, R. S.; JOHNSON, R.; BENESI, A. J.; REINHOLD, V. N.; 1990: Cell-associated oligosaccharides of *Bradyrhizobium* spp. J. Bacteriol. **172**, 136-142.

OTTEN, L.; DE RUFFRAY, P.; MOMOL, E. A.; MOMOL M. T.; BURR, T. J.; 1996: Phylogenetic relationships between *Agrobacterium vitis* isolates and their Ti plasmids. Mol. Plant Microbe Interact. **9**, 782-786.

PU, X.-A.; GOODMAN, R.N.; 1992: Induction of necrogenesis by *Agrobacterium tumefaciens* on grape explants. Physiol. Mol. Plant Pathol. **41**, 241-254.

- -; - -; 1993: Tumor formation by *Agrobacterium tumefaciens* is suppressed by *Agrobacterium radiobacter* HLB-2 on grape plants. Amer. J. Enol. Viticult. **44**, 249-254.

ROY, M. A.; SASSER, M.; 1983: A medium selective for *Agrobacterium tumefaciens* biotype 3 [Abstr.]. Phytopathology **73**, 810.

SAMBROOK, J.; FRITSCH, E. F., MANIATIS, T.; 1989: Molecular Cloning. A Laboratory Manual. 2nd Edition, CSH Press, New York.

STAPHORST, J. L.; VAN ZYL, F. G. H.; STRIJDOM, B. W.; GROENEWOLD, Z. E.; 1985: Agrocin-producing pathogenic and nonpathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. Curr. Microbiol. **12**, 45-52.

STOVER, E. W.; SWARZ, H. J.; BURR T. J.; 1997: Crown gall formation in a diverse collection of *Vitis* genotypes inoculated with *Agrobacterium vitis*. Amer. J. Enol. Viticult. **48**, 26-32.

SÜLE, S.; MOSZAR, J.; BURR, T. J.; 1994: Crown gall resistance in *Vitis* spp. and grapevine rootstocks. Phytopathology **84**, 607-611.

SZEGEDI, E.; NEMETH, J.; 1996: Investigation of grape shoots for

Agrobacterium vitis. Növenyvedelem **32**, 605-609.

WEBSTER, J.; DOS SANTOS, M.; THOMSON, J. A.; 1986: Agrocin-producing *Agrobacterium tumefaciens* strains active against grapevine isolates. Appl. Environ. Microbiol. **52**, 217-219.

WILSON, K.; 1989: Preparation of genomic DNA from bacteria. In: AUSÜBEL, R.; KINSTON, R. E.; MOORE, D. D.; SEIDMAN, J. D.; SMITH, J. A.; STRHUL, K. (Eds.): Current Protocols in Molecular Biology. Greene Publishing and Wilwy Interscience, New York **I**, 241-245.

XIAOYING, C.; WANGNIAN, X.; 1986: A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biotype III strain of *A. tumefaciens* from grapevine. Acta Microbiol. Sin. **26**, 193-199.

YOU, J. F.; XIE, X. M.; CHEN, P. M.; GUO, J. M.; 1990: Control of grape crown gall disease with HLB-2 strain of *Agrobacterium radiobacter*. Chin. J. Biol. Contr. **6**, 35-37.

Received October 29, 1998