Antibiotic Sensitivity of Grapevine: A Comparison Between the Effect of Hygromycin and Kanamycin on Shoot Development of Transgenic 110 Richter Rootstock (*Vitis Berlandieri x Vitis rupestris*)

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The effects of kanamycin and hygromycin on the *in vitro* development of 110 Richter rootstock (*Vitis Berlandieri* x *V. rupestris*) and derived transgenic clones were studied in order to evaluate their suitability as selectable markers for grapevine transformation. Transgenic clones carried both the neomycin phosphotransferase gene (*npt*II) under the nopaline synthase promoter (pNOS) and the hygromycin phosphotransferase (*hpt*) gene under the cauliflower mosaic virus 35S promoter (p35S), which confer the ability to grow in the presence of kanamycin and hygromycin, respectively. Even incorporated at low concentrations, both antibiotics strongly inhibited explant development for the control clone, but hygromycin appeared much more toxic. Whereas growth was stopped with 1 µg/mL hygromycin, 16 µg/mL kanamycin was required to block shoot development. Contrary to the control plants, transgenic clones exhibited a greater tolerance to hygromycin than kanamycin. In some transgenic clones the level of resistance conferred by the *npt*II was not significantly advantageous in terms of their ability to grow in the presence of kanamycin. Conversely, the *hpt* gene provided a strong level of tolerance to hygromycin in all transgenic clones, some clones developing up to 64 µg/mL hygromycin. Strategies for selection of grapevine putative transformants with these two antibiotics are discussed.

Because of the serious limitations of conventional approaches to the genetic improvement of grapevines, the use of biotechnology was intensively studied during the last twenty-five years (Meredith, 1996). Although it is easy to propagate several *Vitis* species (including *V. vinifera* cultivars), *Vitis* interspecific hybrids and *Muscadinia* species by microcuttings or axillary budding, efficient protocols to regenerate through adventitious shoot organogenesis or somatic embryogenesis are not available for many important genotypes (Torregrosa, 1995). Because *in vitro* regeneration systems are a prerequisite for genetic engineering, the grapevine is, like many other perennial species, considered as a recalcitrant plant for biotechnology (Gray & Meredith, 1992).

The first experiments in grapevine transformation were reported by Hemstad & Reisch (1985). By inoculating *Vitis* interspecific hybrids shoots with *A. tumefaciens* or *A. rhizogenes*, these authors could induce the formation of calluses and roots, but no putative transformed plants could be regenerated. In grapevines successful transformation was first achieved by Mullins *et al.* (1990) using a disarmed A. *tumefaciens* vector encoding ß-glucuronidase (*gus*) and neomycin phosphotransferase (*npt*II). These authors could obtain *gus* expressing adventitious buds with two *V. vinifera* and one *V. rupestris* cultivars, but they could obtain transgenic shoots only for the latter. They concluded that the main problem in transforming grapevines is to balance the selection requirement for relatively high concentrations of kanamycin against the inhibitory effects of the antibiotic on shoot development.

Whereas some authors obtained grapevine transformants using the *npt*II resistance gene and kanamycin as selective agent (Mauro *et al.*, 1994; Martinelli & Mandolino, 1994; Franks *et al.*, 1998), others reported the low efficiency and high versatility of kanamycin and succeeded by using hygromycin (Le Gall *et al.*, 1994; Perl *et al.*, 1996). In 85% of the grapevine transformation experiments, however, resistance to kanamycin represents the most commonly used selectable marker.

Among the factors considered as limiting the recovery of transformed plants, determination of the explant sensitivity to antibiotics and the level of resistance provided by corresponding genes of resistance appears to be crucial. However, experiments on the effect of antibiotics on grapevines were rare and focused only on non-transgenic genotypes. Colby & Meredith (1990) studied the effect of kanamycin on the development of shoot tips and calluses of *V. vinifera* and *V. rupestris*. Péros *et al.* (1998) evaluated the response to kanamycin and hygromycin of *V. vinifera* microcuttings cultured on plant growth regulator-free media. Torregrosa *et al.* (1998) studied the effect of hygromycin, kanamycin and phosphinothricin on embryogenic callus development and axillary micropropagation of *Vitis vinifera* L.

Several genetic transformation studies attempted to improve the condition of gene transfer through different methods: *A. tumefaciens* (Martinelli & Mandolino, 1994), *A. rhizogenes* (Nakano *et al.*, 1994), *A. tumefaciens* plus *A. rhizogenes* (Torregrosa & Bouquet, 1997) or biolistic (Kikkert *et al.*, 1996). Actually, the most convenient system consists of co-culture disarmed

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Agrobacterium strains harbouring binary vectors with embryogenic tissues. This recovers transformed plants through 3 main steps: (i) somatic embryogenesis; (ii) induction of axillary or adventitious shoot formation; and (iii) whole plant development. During the regeneration process early selection is generally exerced immediately after or some time co-culture. At this stage necrosis of non-transformed tissues is not suitable because of the risk of exuding deleterious phenolic compounds in the medium. Previous reports on the sensitivity of embryogenic calluses to selective agents (Torregrosa *et al.*, 1998) are useful to define a strategy of selection at an early stage, because the responses of non-transformed tissue determines the selection level. More recently, when putative transformed embryos have been obtained and encouraged to form shoots and plants, stronger selection has had to be applied to avoid false positives. At this stage, in order to determine the highest level of selection, it is crucial to know the sensitivity to selective agents of both transformed and non-transformed buds and shoots.

The purpose of this study was to compare the response to kanamycin and hygromycin of several transgenic clones of grapevines bearing both pNOS-*npt*II and P35S-*hpt* genes during axillary proliferation or plantlet development in order to define the best strategy for late selection of grapevine putative transformants.

MATERIALS AND METHODS

Plant material: Embryogenic calluses of *Vitis Berlandieri* x *V. rupestris* cv. 110 Richter cl. 151 were initiated as previously described by Bouquet *et al.* (1982). Long-term embryonic cultures were established on a half-strength Murashige & Skoog (1962) medium containing 5 µM naphthoxyacetic acid (NOA) or 2,4-dichlorophenoxyacetic acid (2,4-D), 1 µM 6-benzylamino-purine (BAP) and co-cultivated with *A. tumefaciens* strain LBA4404 pKVHG2+ (Brault *et al.*, 1993). The binary pKVGH2+ bears the *npt*II gene under the control of the nopaline synthase promoter, the *hpt* and the *gus* genes under the control of the CaMV 35S ribosomal subunit promoter (p35S) and the gene of the coat protein of the grape chrome mosaic virus gene under the p35S. Putative transformed embryogenic clones were regenerated through somatic embryogenesis as previously described by Le Gall *et al.* (1994). The presence of transgenes was tested by a specific polymerase chain reaction and stable integration, confirmed by Southern blotting (Le Gall *et al.*, 1994). Embryo-derived plantlets were subcultured for two years according to the method of Torregrosa & Bouquet (1995). Explants were single-node microcuttings taken from the intermediate stem part of 3-month-old plantlets. This study was focused on six genotypes: the control 110 Richter cl. 151 (noted VT0) and five transgenic clones (noted VT2, VT3, VT5, VT10 and VT11).

Culture conditions and explant behaviour: The *npt*II (Bevan *et al.*, 1983) and the *hpt* (Van den Elzen *et al.*, 1985) genes encoding neomycin and hygromycin phosphotransferase, respectively, confer to transformed cells, tissues or organs the ability to grow in the presence of kanamycin and hygromycin. Several preliminary experiments with buds cultured in axillary proliferation conditions showed (i) high sensitivity of grapevine to both antibiotics, (ii) high tolerance to cefotaxime, the most commonly used bacteriostatic agent (tested at 0, 100, 200 or 400 µg/mL) and (iii) absence of interaction between cefotaxime and kanamycin or hygromycin. Consequently, the effect of two selective agents, both provided by Sigma®, was further studied using cefotaxime-free media.

Two propagation systems were used in this study: micropropagation through plantlet formation and axillary shoot proliferation. Growth and development of buds cultured under these conditions were previously described (Torregrosa & Bouquet, 1995). For micropropagation microcuttings were transferred to 200 x 25 mm test tubes containing 25 mL of half-strength MS medium, free of plant growth regulators and cultured under 50 $\mu E.s^{-1}.m^{-2}$ light (photoperiod of 15 h). For axillary shoot proliferation, microcuttings were transferred to 100 mm x 10 mm Petri dishes containing 40 mL of B_1 medium (Torregrosa & Bouquet, 1995) and cultured under 15 $\mu E.s^{-1}.m^{-2}$ light (same photoperiod as above). Within 3 months on plant growth regulator-free medium, microcuttings formed a vigorous root system with a single stem composed of 8-12 nodes. In contrast, microcuttings cultured on proliferation media developed in axillary proliferating masses, which were characterised by poor rooting and intense formation of short shoots with very small leaves.

Experimental design: Five concentrations of kanamycin and hygromycin (0, 1, 4, 16 and 64 µg/mL) were tested using a plant growth regulator-free medium. Data for number of roots per plantlet, length of main roots, stem length from the base to the last visible node and number of nodes per stem plantlet were collected after 15, 30, 45 and 60 days in culture. The response of microcuttings to antibiotics was studied through two independent experiments using a split-plot design with the cultivar in main plots and four replicates. An experimental unit was composed of eight explants. Data were subjected to analysis of variance through the general linear model procedure (Proc GLM) of SAS (SAS Institute Inc., 1985). Genotype and antibiotic effects were tested with block x cultivar and block x treatment respectively as error terms. In order to define relationships between variables, a regression analysis was conducted through PROC REG and PROC GLM of SAS.

Four concentrations of kanamycin (0, 10, 25 and 50 µg/mL) and six concentrations of hygromycin (0, 0.5, 1, 2, 5 and 10 µg/mL) were tested using axillary bud proliferation medium. Data for the fresh weight of axillary proliferative masses and number of neo-formed shoots with a length >5 mm were noted 45 days after culture. The response of microcuttings was studied through a complete randomised factorial design with three replicates per treatment, an experimental unit being composed of four explants. Data were analysed through PROC GLM and means compared using the GABRIEL multiple comparison procedure (p=0.01).

RESULTS

Effect of antibiotics on the development of microcuttings: As means and variances from both experiments were similar, pooled data were analysed. On antibiotic-free medium, roots and shoots developed normally for all clones, but with some variability among clones (data not shown). This variability may be due to a somaclonal variation effect, but is more probably the result of the transformation procedure. These genotypic variations could influence the response of different transgenic clones to antibiotics, independently of the direct effect of transgenes.

When antibiotics were incorporated into the medium, results indicated highly significant effects of genotype and antibiotic on explant development (Table 1). A significant interaction between genotype and antibiotic was found, indicating a differential response among genotypes subjected to antibiotics. Consequently, the ranking of different clones changed according to antibiotic level (data not shown). For instance, the VT2 clone, which had a greater capacity for root and shoot development than VT5 at low antibiotic levels, exhibited a lower antibiotic resistance than VT5 at higher concentrations. The VT10 and VT11 clones, which has the lowest root development capacities on control medium, showed the best capacities of root elongation with higher hygromycin concentrations. Furthermore, the resistance level of a clone to hygromycin and kanamycin could be different. For instance, VT5 exhibited high and similar levels of resistance to both antibiotics. Root and shoot development were not significantly reduced for up to 4 µg/mL kanamycin or hygromycin. In contrast, VT10, which resisted up to 16 µg/mL hygromycin, did not show any level of resistance to kanamycin compared to VT0 (Fig. 1).

Otherwise, the effects of both antibiotics appeared very different. On the control clone, kanamycin proved much less phytotoxic than hygromycin (Fig. 1). At 1 µg/mL kanamycin, rhizogenesis and root elongation were lightly stimulated, but shoot development was not modified. At 4 µg/mL kanamycin root elongation ceased and rhizogenesis and stem elongation were strongly reduced. At 16 µg/mL kanamycin, rhizogenesis was completely inhibited and stems were stunted with small leaves that turned yellow. At 64 µg/mL kanamycin, buds produced very weak stems bearing chlorotic leaves showing necrosis and leafroll. At 1 and 4 µg/mL hygromycin, rhizogenesis was already terminated and bud development resulted in rare, very weak stems, similar to those produced at 64 µg/mL kanamycin. At 16 µg/mL hygromycin, though root and stem growth were completely stopped, explants remained apparently well conditioned. At 64 µg/mL explants did not develop at all and rapidly turned brown.

Transgenic clones exhibited a significant level of resistance to the tested antibiotics, but contrary to the control clone, showed a markedly stronger resistance to hygromycin than kanamycin (Fig. 2). Rhizogenesis was not altered by up to 4 µg/mL kanamycin or hygromycin and was reduced at 16 µg/mL kanamycin or hygromycin. Rhizogenesis was strongly inhibited by 64 µg/mL hygromycin, and blocked at 64 µg/mL kanamycin. Root elongation response varied in a similar way, but with a greater inhibition effect of kanamycin. At 4 µg/mL kanamycin, root length was similar to that obtained at 16 µg/mL hygromycin. Regarding stem development, hygromycin caused little effect up to 4 µg/mL; at 16 µg/mL and 64 µg/mL there was a marked inhibition. In contrast, 1 µg/mL to 64 µg/mL kanamycin showed a gradual inhibition of shoot development.

Correlation analysis performed on both control and transgenic clones (Table 2) indicated that, in the absence of antibiotics, (i) no correlation existed between the root number and the root length, (ii) rhizogenesis did not affect shoot development and (iii) the number of nodes formed was positively correlated with stem length. In the presence of antibiotics, levels of correlation increased, indicating that the more the plantlets were stressed, the more the variables varied in the same way.

Effect of antibiotics on the development of axillary proliferative masses: When incorporated in the axillary proliferative medium, antibiotics induced similar effects to those in a plant growth regulator-free medium (Fig. 3). In the non-transgenic clone, 10 µg/mL kanamycin effectively reduced axillary bud development, resulting in a strong decrease in shoot initiation and growth. At 25 µg/mL kanamycin, explants

TABLE 1
Effect of clone and antibiotic on *in vitro* development of grapevine microcuttings established on a plant growth regulator-free medium (data collected after 60 days in culture).

Source of variation	df	Mean squares[a]			
Kanamycin		Number of roots	Root length (mm)	Number of nodes	Stem length (mm)
Clone	5	019.91***	021331.59***	0189.04***	008613.33**
Kana level	4	114.80***	119672.63***	1070.90***	110272.63***
Clone*level	20	016.20***	005081.81***	0035.49***	004067.17***
Error	888	000.64	000148.81	0002.29	000174.46
Hygromycin					
Clone	5	046.26***	22233.45***	0675.33***	045664.89***
Hygro level	4	058.91***	64009.36***	1717.08***	122328.24***
Clone*level	20	006.65***	07155.05***	0104.28***	007959.84***
Error	888	000.71	00260.46	0005.11	000217.74

[a]The experimental design was a split-plot with cultivar as main plot. The effects of clone (6 clones) and antibiotic (kanamycin or hygromycin at 0, 1, 4, 16, or 64 µg/mL) were tested using respectively block* clone and block* level as error terms. *, **, ***, ns: F test significant at p=0.05, p=0.001 or not significant.

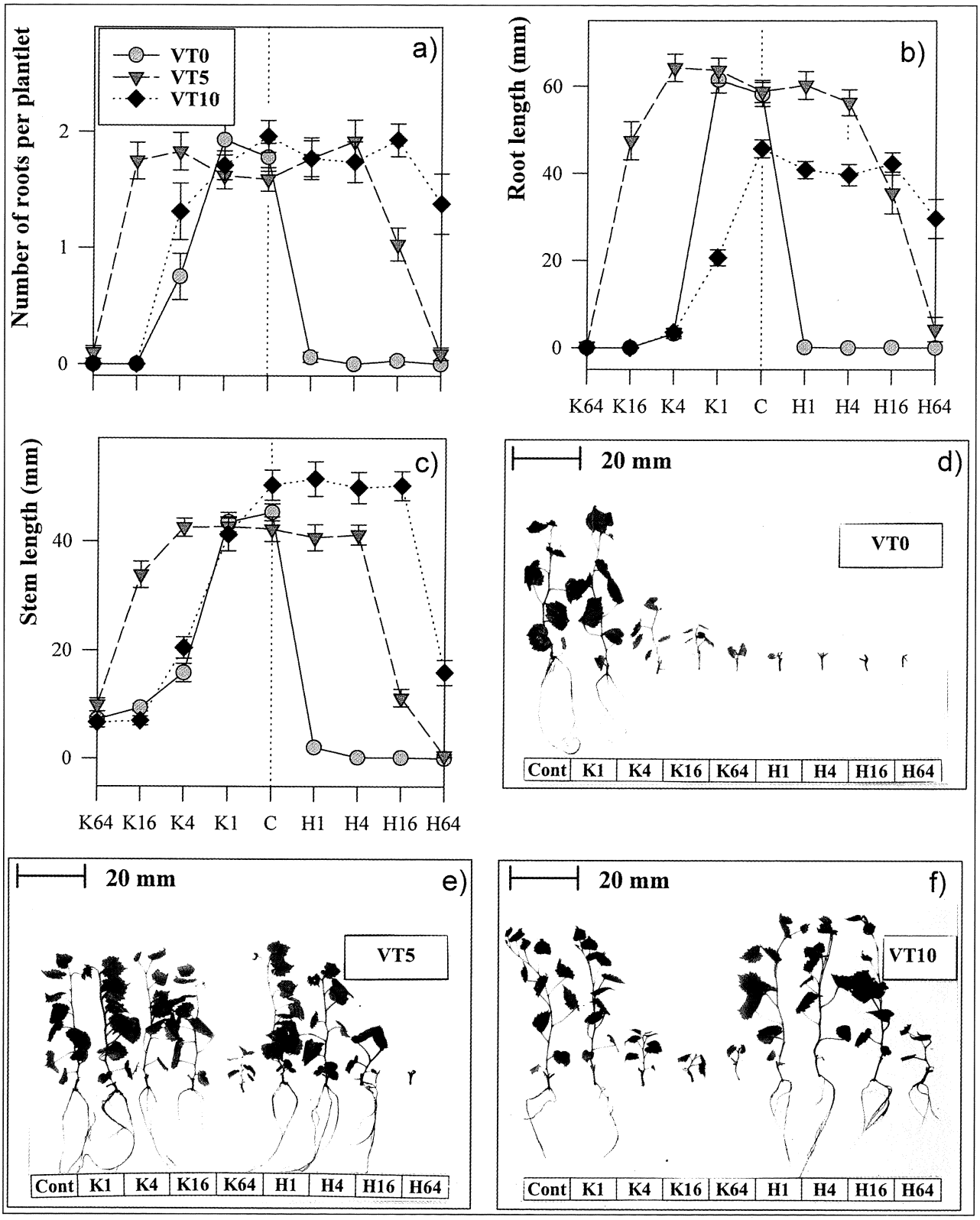

FIGURE 1

Effect of kanamycin and hygromycin at 0 (Cont), 1 (K1 or H1), 4 (K4 or H4), 16 (K16 or H16) and 64 µg/mL (K64 or H64) on the development of VT0, VT5 and VT10 microcuttings after 60 days in culture: a), b) and c) are compared data for number of roots, root length and stem length (vertical bars correspond to ± SE); d), e) and f) show the level of development of the 3 genotypes.

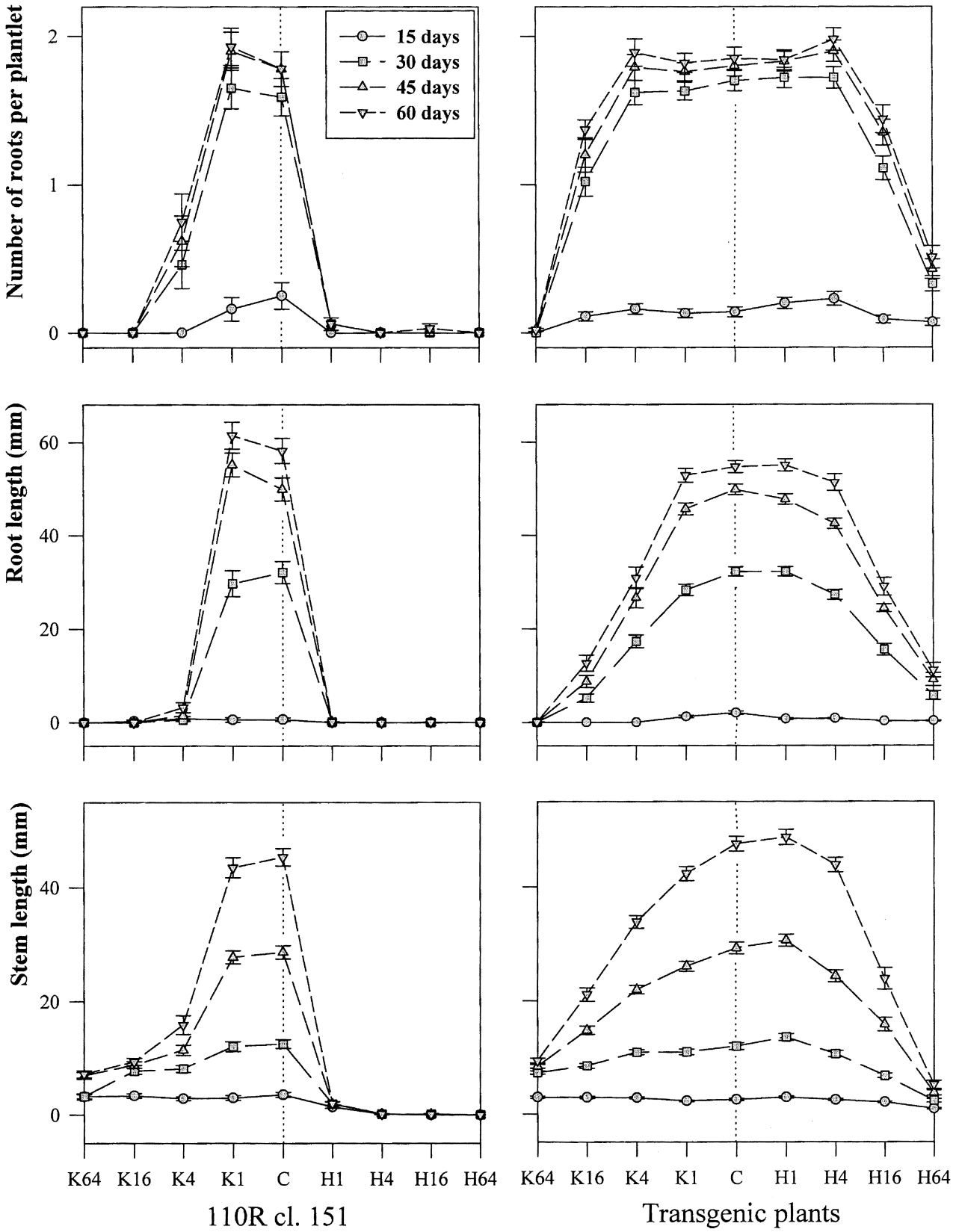

FIGURE 2

In vitro response of the control (11OR cl. 151, on the left) and transgenic clones (pooled data from VT2, VT3, VT5, VT10 and VT11, on the right) to the addition of kanamycin or hygromycin (1, 4, 16 or 64 µg/mL) to plant regulator-free medium. Data accumulated at 15, 30, 45 and 60 days (vertical bars correspond to ± SE).

TABLE 2

Correlations between number of roots (NR), root length (RL), stem length (SL) and number of nodes (NN) of plantlets established on a plant growth regulator-free medium (data collected after 60 days in culture).

Comparison	Clone[a]	Antibiotic level [b]								
		Control	K1	K4	K16	K64	H1	H4	H16	H64
NR *vs* RL	VTO	-0.14 (0.0452)c	-0.36 (0.040)	0.46 (0.0008)	–	–	1.00 (0.0001)	–	–	–
	VT	-0.24 (0.0016)	-0.22 (0.0001)	-0.09 (0.2663)	0.34 (0.0001)	1.00 (0.0001)	0.02 (0.7834)	-0.18 (0.0176)	0.28 (0.0004)	0.61 (0.0001)
NR *vs* SL	VTO	0.23 (0.208)	0.31 (0.0790)	0.56 (0.0006)	–	–	0.44 (0.0011)	–	–	–
	VT	0.32 (0.0001)	0.33 (0.0001)	0.36 (0.001)	0.65 (0.0001)	0.42 (0.0001)	0.45 (0.0001)	0.32 (0.0001)	0.42 (0.0001)	0.65 (0.00001)
RL *vs* SL	VTO	0.15 (0.414)	0.21 (0.0243)	0.62 (0.0002)	–	–	0.42 (0.0013)	–	–	–
	VT	0.13 (0.0931)	0.34 (0.0001)	0.54 (0.0001)	0.63 (0.0001)	0.42 (0.0001)	0.28 (0.0186)	0.25 (0.0013)	0.56 (0.0001)	0.74 (0.0001)
SL *vs* NN	VTO	0.65 (0.0001)	0.65 (0.0001)	0.87 (0.0001)	0.80 (0.0001)	0.84 (0.0001)	0.74 (0.0001)	–	–	–
	VT	0.77 (0.0001)	0.87 (0.0001)	0.91 (0.0001)	0.93 (0.0001)	0.83 (0.00001)	0.79 (0.0001)	0.57 (0.006)	0.95 (0.0001)	0.91 (0.0001)

[a]VTO corresponds to the control 110R cl. 151 and VT to the pooled data from the 5 transgenic clones VT2, VT3, VT5, VT10 and VT11.
[b]Kanamycin and hygromycin were incorporated in a plant regulator-free medium.
[c] Corresponds to the p-value.
(–) Means equal to zero.

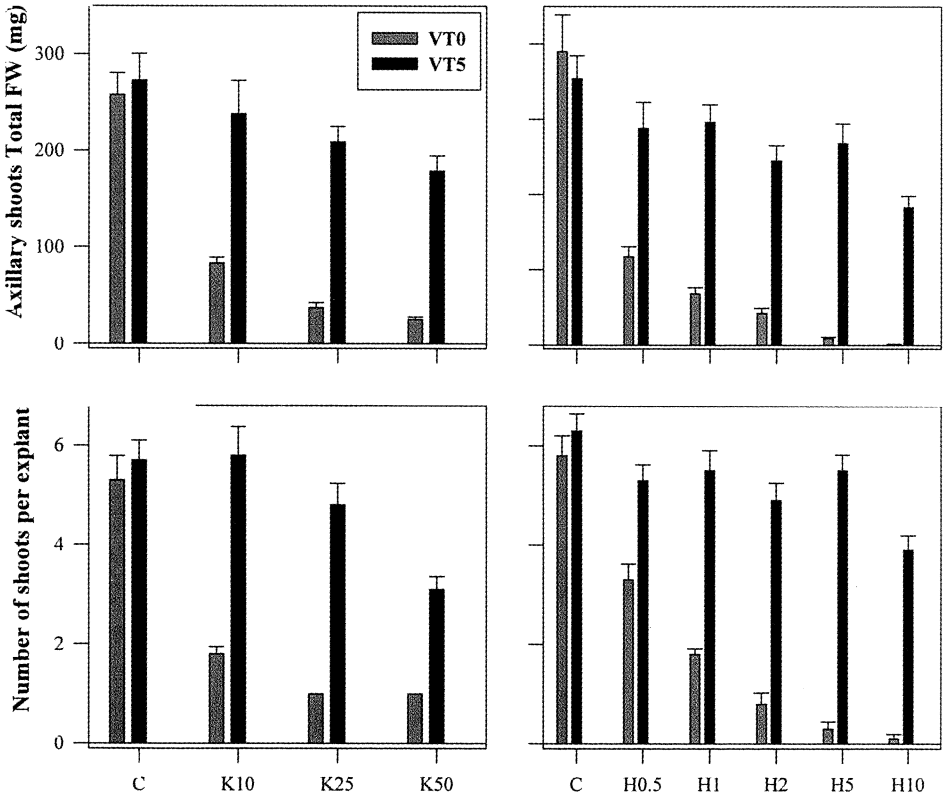

FIGURE 3

In vitro response of transgenic clone (VT5) compared to the control to the addition of kanamycin (10, 25 or 50 µg/mL) or hygromycin (0.5, 1, 2, 5 or 10 µg/mL) in axillary proliferation medium. Data collected after 45 days in culture (vertical bars correspond to ± SE).

produced stunted shoots bearing small distorted leaves, which turned yellow. At 50 µg/mL kanamycin, a single very stunted shoot with reddening leaves was initiated, but the explants remained green. With hygromycin, the development of the non-transgenic clone was strongly reduced at 1 µg/mL. From 2 µg/mL, hygromycin stopped axillary bud development and explants became progressively brown. Therefore the effect of kanamycin is very different from that of hygromycin: (i) compared to hygromycin, kanamycin proved less effective in reducing the development of explants; (ii) whatever the concentration employed, kanamycin could not prevent shoot emergence; and (iii) kanamycin was less phytotoxic than hygromycin, which might induce severe necrosis. The levels of resistance exhibited by VT5 (Fig. 3) and also by other transgenic clones (data not shown) were similar to those observed with plant regulator-free medium, indicating the absence of interaction between the effects of antibiotics and the effects of plant growth regulators.

When cultured onto an axillary proliferation medium, micro-cuttings did not develop any root system. The medium supplied the explant directly with nutrients through the callus initiated at the explant base. These observations strengthen the hypothesis that selective antibiotics cause a general phytotoxic effect, which do not result from a specific rhizogenesis inhibition.

DISCUSSION

Axillary buds appeared much more sensitive than embryogenic tissue, irrespective of the medium. Perl *et al.* (1996) reported that kanamycin (50-500 µg/mL) was unable to inhibit embryo regeneration. Torregrosa *et al.* (1998) indicated that according to the genotype, 1.25-2.5 µg/mL hygromycin or 80-100 µg/mL kanamycin were required to significantly inhibit callus proliferation. In this study exillary buds of 110 Richter cl. 151 proved sensitive to both antibiotics as well, but with a ten-fold greater sensitivity to hygromycin than to kanamycin. These results are consistent with those reported by Colby & Meredith (1990) and by Péros *et al.* (1998). The former, studying the effect of kanamycin on three *V. vinifera* cultivars and the rootstock *V. rupestris* cv. Du Lot, observed that (i) the percentage of micro-cuttings forming roots decreased at 5 µg/mL and was completely inhibited at 10 µg/mL, and that (ii) axillary shoot formation was strongly reduced at 4 µg/mL and completely stopped at 7 µg/mL. The latter authors, studying the effect of kanamycin and hygromycin on several *V. vinifera* cultivars, reported the high sensitivity of grapevines to both antibiotics. In their study hygromycin was lethal at 0.8 µg/mL, whereas this effect was only observed at 4 µg/mL of kanamycin.

Other woody species have proved to be less sensitive to antibiotics than grapevines. For instance, development of shoot cultures of *Ulmus procera* (Fenning *et al.*, 1993) and *Malus x domestica* Borkh. (Yepes & Aldwinckle, 1994b) was possible in the presence of 25 µg/mL kanamycin. Therefore, for species in which kanamycin presents a high efficiency for selection, high concentrations can be employed. For instance, Fillati *et al.* (1987) used 60 µg/mL to select neoformations in *Populus* and James *et al.* (1989) used regeneration medium containing 200 µg/mL kanamycin with *Malus pumila*.

Despite the higher phytotoxicity of hygromycin, transgenic clones bearing p35S-*hpt* and pNOS-*npt*II genes exhibited better tolerance to hygromycin than kanamycin. Because of insufficient resistance levels conferred by the pNOS-*npt*II gene, some transgenic clones showed a poor selective advantage compared to the control clone in the presence of kanamycin. Difficulties reported by several researchers in transforming grapevines (Guellec *et al.*, 1990; Mullins *et al.*, 1990; Mauro *et al.*, 1994) could result from the lack of efficiency of kanamycin-resistant genes when controlled by a pNOS promoter rather than from high grapevines sensitivity to kanamycin.

Problems of selection using kanamycin resistance were reported for other plants as well. For instance, Norelli & Aldwinckle (1993) studied the effect of four aminoglycosides antibiotics (neomycin, kanamycin, geneticin and paromomycin) on adventitious shoot formation from *npt*II-transgenic and non-transgenic apples. They found that the *npt*II gene did not give any growth advantage to the transgenic clones when kanamycin or geneticin were incorporated into the culture medium. Consequently, kanamycin and geneticin appeared unsuitable for selection, while neomycin and paromomycin proved efficient selective agents. In agreement with this report, Mauro *et al.* (1994) succeeded in grapevine transformation of two rootstock and one *V. vinifera* cultivar with the pNOS-*npt*II gene, but using paromomycin as selective agent instead of kanamycin.

Inadequate efficiency of the pNOS-*npt*II gene could have several causes: (i) the number of functional gene copies integrated in the plant genome, (ii) the level of gene expression, which depends on insert localisation and promoter-specific activity, and (iii) efficiency of expression products (NPT). However, because the binary pKVGH 2+ carries both *npt*II and *hpt* genes and transgenic clones were checked for the presence of the *npt*II gene, we can expect that the low efficiency of pNOS-*npt*II results from the poor promoter activity or product efficiency.

Lack of efficiency of the pNOS-*npt*II gene can hinder any selection strategy with kanamycin: i.e. (i) preselection on kanamycin immediately after transformation followed by a period without selection as proposed by James *et al.* (1989) for apples; (ii) permanent selection of putative transformed tissues and regenerative structures; or (iii) a strategy of delayed selection, as proposed by Yepes & Aldwinckle (1994a). In the selection scheme of recovering transgenic shoots, an appropriate selectable marker has to be chosen. This study demonstrated that the choice depends not only on tissue sensitivity to the selective agent, but also on the level of tolerance conferred by the corresponding resistance gene.

CONCLUSIONS

In the described system, the p35S-*hpt* gene appeared more suitable than pNOS-*npt*II because of the great tolerance conferred to transgenic buds and the high sensitivity to hygromycin of grapevine. Consequently, hygromycin appears an appropriate and alternative selective agent to kanamycin for delayed selection of putative transformed buds and shoots. Because of the high sensitivity of the grapevine, very low levels of hygromycin (1 to 4 µg/mL) have to be used to avoid excessive necrosis of surrounding tissues.

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