Vitis 29, 151—158 (1990)

Department of Viticulture and Enology, University of California, Davis, CA, U.S.A.

Adventitious bud formation in leaf explants of some grapevine rootstock and scion cultivars

by

F. A. TANG and M. G. MULLINS

Die Bildung von Adventivknospen an Blattexplantaten einiger Unterlags- und Edelreissorten der Rebe

Zusammenfassung: Adventivknospen bildeten sich am häufigsten an Blattexplantaten der Sorten Rupestris St. George, Thompson Seedless und Niagara (ca. 35—50 %), in mittlerer Häufigkeit bei Ramsey, Cabernet Sauvignon, Chardonnay und Concord (ca. 25—30 %), am wenigsten häufig bei 110-R und ARG 1 (20 %). Explantate von Blattstielen waren im allgemeinen regenerationsfähiger als solche aus Blattspreiten. Die Art und Weise, in der die Mutterpflanzen kultiviert wurden (Klimakammer oder *in vitro*), wirkte sich später nicht auf die Bildung von Adventivknospen aus. Benzyladenin (10 µM) und α-Naphthylessigsäure (0,05—0,10 µM) waren in Verbindung mit Cytokinin und Auxin für die Bildung von Adventivknospen am wirksamsten. An Blattspreiten-Explantaten wurden mehr Knospen gebildet (1—12) als an Blattstiel-Explantaten (1—6). Ein gleichbleibender Einfluß der Behandlung auf die Anzahl der Knospen je Explantat lag jedoch nicht vor. Der maßgebliche Faktor war der Prozentsatz der Explantate, die überhaupt Adventivknospen ausbildeten.

Key words: bud, tissue culture, explant, leaf, lamina, petiole, regeneration, genotype, variety of vine, growth regulator.

Introduction

Regeneration of grapevines *in vitro* by somatic embryogenesis is a well established procedure (MULLINS and SRINIVASAN 1976; SRINIVASAN and MULLINS 1980), as is the fragmented apex technique of BARLASS and SKENE (1978), but efficient methods for regeneration of grapevines by adventitious bud formation in leaf, stem or root explants are still in the process of development.

Adventitious bud formation *in vitro* in the grapevine was first reported by FAVRE (1976, 1977). The fragmented apex technique of grapevine propagation involves the formation of adventitious buds by leaf primordial fragments (BARLASS and SKENE 1980 a and b, 1981), and organogenesis in callus of *Vitis* spp. was described by RAJASEKARAN and MULLINS (1981). Explants from the hypocotyls of somatic embryos of several cultivars of *Vitis vinifera* form adventitious buds with high frequency (VILAPLANA and MULLINS 1989).

The use of leaf material, lamina or petioles, as initial explants for regeneration of grapevines by the route of adventitious buds, was described by REISCH *et al.* (1989). In contemporaneous research in this laboratory it was also found that lamina and petiole tissues from young unexpanded leaves of some grapevine cultivars have the capacity to form adventitious buds when grown on media containing both auxin and cytokinin. However, bud formation was sporadic, and this led to the present research in which a systematic study was made of factors affecting adventitious bud formation on 10 genotypes representing wine grapes, table grapes and rootstocks.

Materials and methods

Explants from grapevines grown in a controlled-environment chamber

Grapevines of 10 genotypes (Table 1) were propagated from cool-stored (3—5 °C) hardwood cuttings. Cutting material from certified mother vines was obtained from the collections of the Department of Viticulture and Enology and the Foundation Plant Materials Service, University of California, Davis. The vines were grown in pots (21 cm in diameter) containing a commercial potting-mix (Supersoil, McAllelan Co.; South San Francisco, California) in a controlled environment chamber (26 °C day, 20 °C night, 14 h photoperiod). Light was provided by a mixture of incandescent and cool white fluorescent lamps. The luminous flux density at the level of shoot tips was 230—250 µmol $m^{-2}sec^{-1}$. Plants were fertilized twice monthly with a commercial liquid formulation (NPK 23:19:17). Explants were harvested after 2 months. Young leaves (less than 5 cm in width) were excised with petioles attached and were surface-sterilized by shaking (20 min) with a sodium hypochlorite solution (0.5 %) containing 0.1 % Tween 80 wetting agent. After four rinses in sterile distilled water, the leaf laminae were cut into 3 mm × 6 mm fragments and petioles were cut into segments (each 2—3 mm in length) for culture.

Table 1

Plant materials: Studies on effects of genotype on adventitious bud formation in lamina and petiole explants

Pflanzenmaterial: Untersuchungen über den Einfluß des Genotyps auf die Bildung von Adventivknospen an Blattspreiten- und Blattstielexplantaten

Species	Cultivar	Synonyms	Comment
Vitis rupestris	St. George	Rupestris du Lot	rootstock, phylloxera resistant
Vitis champini	Dog Ridge	—	rootstock, nematode resistant
Vitis champini	Ramsey	Salt Creek	rootstock, nematode resistant
Vitis berlandieri × *V. rupestris*	110-R	110-Richter	rootstock, phylloxera resistant
Vitis vinifera × *V. rupestris*	AxR#1	Ganzin-1 ARG-1	rootstock
Vitis vinifera	Cabernet Sauvignon	—	wine grape (red)
Vitis vinifera	Chardonnay	—	wine grape (white)
Vitis vinifera	Thompson Seedless	Sultana Sultanina	wine (white), table and raisin grape
Vitis × *labruscana*	Niagara	—	table grape
Vitis × *labruscana*	Concord	—	wine grape, juice grape

Explants from grapevines grown *in vitro*

Single node explants (length 1—2 cm) were collected from field-grown vines of Cabernet Sauvignon and Chardonnay. Nodal explants of Thompson Seedless, Niagara

and Concord were from greenhouse-grown material, and those of St. George, Ramsey, Dog Ridge, AxR # 1 and 110-R were from vines grown in the controlled-environment chamber. Surface sterilization was as before. Explants were cultured on an agar-based medium (0.65 %, Phytagar®, Gibco Laboratories, Grand Island, New York), i.e. basal medium of NITSCH and NITSCH (1969) supplemented with sucrose (3 %) and γ-indolebutyric acid (IBA, 5 μM). The pH was adjusted to 5.7 using 0.1 N KOH before autoclaving (15 min, 121 °C). The nodal explants were cultured in clear plastic containers (Gibco Laboratories, Grand Island, New York) in a controlled-environment chamber (26 °C day, 20 °C night, 14 h photoperiod). Illumination was by cool white fluorescent tubes (40 W). The luminous flux density was approximately 40 μmol $m^{-2}sec^{-1}$. Regrowth from the initial nodal explants was subcultured at 2—3 monthly intervals. Leaves (less than 5 mm in width) from these cultures were used in experiments on adventitious bud formation. Laminae were cut into two or three fragments and petioles were made into segments (1—2 mm in length).

Treatments and growing conditions for adventitious bud formation

Explants (laminar, petiolar) from mother plants of each of the 10 genotypes, grown either *in vitro* or in a controlled-environment chamber, were cultured on a modified NITSCH and NITSCH (1969) medium, as before, containing benzyladenine (BA, 10 μM) either alone or in combination with α-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). The concentrations of both auxins were as follows: 0, 0.05, 0.10, 0.50 and 1.00 μM.

Explants were cultured in 25 ml of agar-based medium in Petri dishes (1.5 cm × 20 cm). Leaf explants were grown with their abaxial surfaces uppermost; petiole explants were placed horizontally. There were 20 explants per plate and 2 plates for each hormonal treatment. Cultures were grown in darkness for 4—8 weeks and were then transferred to an illuminated growth chamber (temperatures, photoperiod, luminous flux density as before). Observations on adventitious bud formation in explants were made periodically during the next 4—6 weeks. Final records on adventitious bud formation were made after 8 weeks. Further experimental details are given with 'Results'.

Results

Effects of genotype on adventitious bud formation

Adventitious bud formation occurred in both lamina and petiole explants in all of the 10 genotypes. There were three distinct stages of bud development. Stage I comprised the formation of rudimentary apical meristems (Fig. 1). These dome-like structures were different in color and form from the callus from which they arose, but were without leaf primordia. In Stage II there were discernable leaf primordia (Fig. 2) and in Stage III (Fig. 3) there was elongation of the adventitious bud axis to form a leafy shoot.

In the genotypes 110-R and AxR # 1, buds remained at Stage I and could not be induced to differentiate further by subculture, cold-treatment (4 °C for 4 weeks) or treatment with gibberellic acid (1 μM). In these experiments the explants containing Stage I buds reverted to callus production or turned brown and died. In the next stage, Stage II, there was discernable elongation of meristems and formation of leaf primor-

dia (Fig. 2). In Stage III there was elongation of the adventitious bud axis to produce a leafy shoot (Fig. 3). With the exception of Concord, explants which produced Stage II buds advanced subsequently to Stage III (Table 2). From Stage II onwards, buds from leaf and petiole explants were readily induced to form adventitious roots (Fig. 4) by transfer to hormone-free media or to media containing IBA (5 μM).

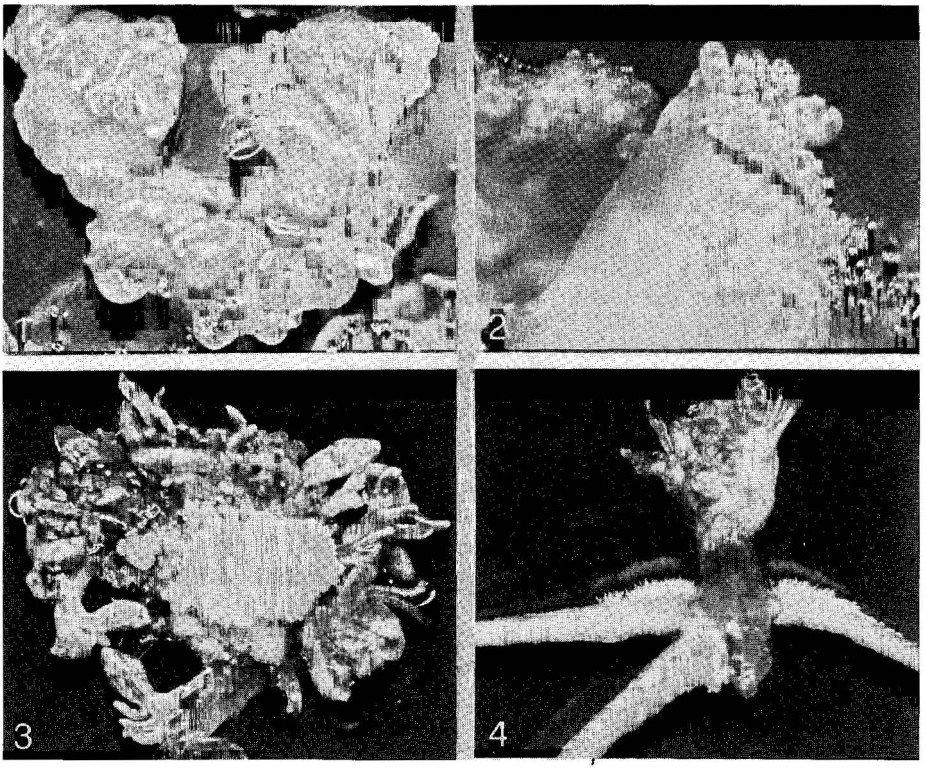

Fig. 1: Stage I. Rudimentary adventitious buds, dome-like structures are visible but leaf primordia are absent. Lamina explant of Cabernet Sauvignon photographed after 6 weeks in darkness, i.e. at point of transfer to light.

Fig. 2: Stage II. Elongation of incipient adventitious buds. Leaf primordia are visible. Lamina explant of Ramsey photographed after 12 weeks of culture.

Fig. 3: Stage III. Petiole explant of Chardonnay showing multiple leafy shoots. Photographed after 12 weeks of culture.

Fig. 4: Plantlet of Chardonnay. Note adventitious roots and root hairs. Photographed 3 weeks after transfer of a Stage III leafy shoot to rooting medium containing IBA (5 μM).

Abb. 1: Stadium I. Rudimentäre Adventivknospen; es sind kuppelförmige Strukturen sichtbar, Blattprimordien fehlen jedoch. Blattspreiten-Explantat von Cabernet Sauvignon nach 6wöchiger Dunkelkultur, d.h. beim Wechsel zur Lichtkultur.

Abb. 2: Stadium II. Streckung der frühen Adventivknospen. Die Blattprimordien sind sichtbar. Blattspreiten-Explantat von Ramsey nach 12wöchiger Kultur.

Abb. 3: Stadium III. Blattstiel-Explantat von Chardonnay mit zahlreichen beblätterten Sprossen nach 12wöchiger Kultur.

Abb. 4: Chardonnay-Pflänzchen 3 Wochen nach der Übertragung eines beblätterten Sprosses (Stadium III) auf Bewurzelungsmedium mit 5 μM IBA. Man beachte die Adventivwurzeln mit den Wurzelhaaren.

Effects of genotype, explant type, culture medium and treatment of mother plants

The number of adventitious buds formed on petiole explants varied from 1 to 6. These buds arose from callus which grew at the cut-ends of the explants. Bud formation was more prolific in lamina explants (1—12) and was associated with callus which grew at the periphery of the explants close to the cut-ends of veins or midribs.

There were no clear effects of genotype, culture media, or of the source of mother plants (cultivated in a growth chamber or *in vitro*), on the numbers of buds per explant. Numbers of buds were similar over a wide range of treatments. In contrast, the most pronounced treatment effects were on the numbers of explants which produced adventitious buds. Within each Petri dish, bud formation occurred in 20—50 % of the explants. The remaining explants produced callus, but adventitious bud primordia were not detected. Attempts to induce adventitious buds in these non-regenerating explants by subculture were unsuccessful.

Table 2

Summary of effects of genotype on formation of adventitious buds and shoots in lamina and petiole explants

Zusammenfassung: Einfluß des Genotyps auf die Bildung von Adventivknospen und Sprossen an Blattspreiten- und Blattstiel-Explantaten

Genotype	Stage I	Stage II	Stage III
	Formation of rudimentary adventitious buds (Fig. 1)	Formation of well-differentiated adventitious buds (Fig. 2)	Regeneration of shoots and plants (Figs. 3, 4)
St. George	+	+	+
Dog Ridge	+	+	+
Ramsey	+	+	−
110-R	+	−	−
AxR#1	+	−	−
Cabernet Sauvignon	+	+	+
Chardonnay	+	+	+
Thompson Seedless	+	+	+
Niagara	+	+	+
Concord	+	+	−

Explants were cultured for up to 16 weeks on NITSCH and NITSCH (1969) basal medium containing BA (10 μM) and either NAA (0.05 μM) or NAA (0.1 μM).

The highest frequency of explants producing adventitious buds occurred in St. George, Thompson Seedless and Niagara (approx. 35—50 %). Dog Ridge, Ramsey, Cabernet Sauvignon, Chardonnay and Concord were intermediate (approx. 25—30 %) and bud formation was least frequent in explants of 110-R and AxR # 1 (approx. 20 %).

The response of explants to growth regulators was clear. Media containing BA (10 μM) and NAA (0.05—0.10 μM) were the most effective for induction of adventitious buds in all genotypes and in all types of explants (Table 3). Explants treated with 2,4-D in combination with BA produced callus only. With two exceptions, Cabernet Sauvignon and Chardonnay, the proportion of explants which formed buds was greater in

petiolar explants than in laminar explants. There were no consistent effects on adventitious bud formation by explants of the growing conditions of the mother plant. Regenerative explants were derived equally from growth chamber-propagated mother plants and from mother plants grown *in vitro*.

Table 3

Effects of genotype, source of mother plants, source of explants and culture medium on adventitious bud formation: Summary of the most effective treatment combinations for regeneration

Einfluß von Genotyp, Herkunft der Mutterpflanzen, Art des Explantats und des Kulturmediums auf die Bildung von Adventivknospen: Zusammenfassung der für die Regeneration wirksamsten Behandlungskombinationen

Genotype	Max. % explants[1])	Source of mother plants forming buds	Source of explants	Medium (μM)
St. George	45	growth chamber	petiole	BA: 10, NAA: 0.05
Dog Ridge	31	*in vitro*	petiole	BA: 10, NAA: 0.05
Ramsey	29	growth chamber	petiole	BA: 10, NAA: 0.10
110-R	19	*in vitro*	petiole	BA: 10, NAA: 0.05
AxR#1	21	growth chamber	petiole	BA: 10, NAA: 0.10
Cabernet Sauvignon	26	growth chamber	leaf	BA: 10, NAA: 0.10
Chardonnay	31	*in vitro*	leaf	BA: 10, NAA: 0.10
Thompson Seedless	52	growth chamber	petiole	BA: 10, NAA: 0.05
Niagara	36	*in vitro*	petiole	BA: 10, NAA: 0.05
Concord	27	*in vitro*	petiole	BA: 10, NAA: 0.10

[1]) Mean maximum values from two experiments. Percentage of explants forming one or more adventitious buds after 16 weeks of culture. There were 80 explants per treatment.

Discussion

The experiments described here were prompted by the need for an efficient, simple regeneration system in grapevines, based on formation of adventitious buds, for use in research on genetic transformation. Earlier work on *Agrobacterium*-mediated transformation using the fragmented apex technique of BARLASS and SKENE (1978) was unsuccessful (BENNETT 1988).

In the present research, there were no consistent effects of treatments on bud number per explant. The overriding factor in these experiments was the proportion of explants which formed adventitious buds. Even in the most highly regenerative group of genotypes (St. George, Thompson Seedless and Niagara) a maximum of 35—50 % of explants gave rise to buds. The remaining explants growing in the same Petri dishes on the same culture media formed only callus. This variation among explants was not attributable to treatment of the mother plant or to the type of explant (Table 3). Further, it is unlikely that variation in the responses of explants *in vitro* resides in the culture conditions because the combination of BA (10 μM) and NAA, within the range of 0.05—1.0 μM, was an effective treatment for induction of adventitious buds over a wide range of other treatments (Table 3).

The reasons why these carefully selected lamina and petiole explants were so variable in regenerative competence is unknown. One possibility is that the capacity for

adventitious bud formation, which is manifested strongly by shoot apical fragments (BARLASS and SKENE 1978), is negatively correlated with leaf age or leaf size. The leaves used in the present research, although unexpanded and less than 5 cm in width, may have had only residual competence to form adventitious buds.

The observation that there are differences among genotypes in the frequency of adventitious bud formation is consistent with experience in many other species. It is noteworthy that explants of all genotypes produced Stage I adventitious buds. Except for 110-R and AxR # 1, the Stage I buds then proceeded to grow into well formed adventitious buds (Stage II). The next stage (III), elongation of buds and formation of leafy shoots, occurred in only 6 of the 10 genotypes. These results suggest that the induction process occurred in all genotypes and that limitations to further development were primarily at the level of differentiation. The nature of these limitations is unknown, but gibberellin and abscisic acid do not seem to be implicated because exogenous GA_3 and chilling did not promote the development of Stage I or II buds into Stage III buds.

In some woody perennial fruit plants, micropropagation *in vitro* seems to enhance the competence of explants to form buds (WELANDER 1988 (apple); JAMES *et al.* 1988 (apple)) or adventitious roots (SRISKANDARAJAH *et al.* 1982 (apple); BALERIOLA-LUCAS and MULLINS 1984 (prune)). In grapevines, however, explants taken from mother plants grown *in vitro* did not differ in adventitious bud formation from those taken from mother plants grown in a controlled-environment chamber.

To summarize, the grapevine remains a difficult subject for regeneration *in vitro* by the route of adventitious bud formation from leaf explants. Some progress has been made in defining the conditions for regeneration in petiole and lamina explants, but the frequency of bud formation is still markedly lower than in commonly-used experimental plants such as tobacco or petunia. Further improvement is required to achieve the aim of an efficient regeneration system for use in genetic transformation research. The main problem in grapevines seems to be at the level of selection of explants with the competence to regenerate rather than with the identification of specific culture conditions for induction of adventitious bud primordia.

Summary

Adventitious bud formation in leaf explants was of highest frequency in St. George, Thompson Seedless and Niagara (approx. 35—50 %), intermediate in Ramsey, Cabernet Sauvignon, Chardonnay and Concord (approx. 25—30 %), and lowest in 110-R and AxR # 1 (20 %). Petiole explants were generally more highly regenerative than lamina explants. The mode of cultivation of mother plants (growth chamber or *in vitro*) did not affect subsequent adventitious bud formation. Benzyladenine (BA, 10 µM) and α-naphthaleneacetic acid (NAA, 0.05—0.10 µM) were the most effective combination of cytokinin and auxin for adventitious bud formation. Numbers of buds were greater in laminar explants (1—12) than in petiolar explants (1—6), but there were no consistent treatment effects on number of buds per explant. The predominant factor was the proportion of explants forming adventitious buds.

Acknowledgements

This research was supported by the Amerine Chair (endowed by the Ernest Gallo Foundation) and by Pope Vineyards, Pope Valley, California.

References

BALERIOLA-LUCAS, C.; MULLINS, M. G.; 1984: Micropropagation of two French prune cultivars (*Prunus domestica* L.). Agronomie **4**, 473—478.

BARLASS, M.; SKENE, K. G. M.; 1978: *In vitro* propagation of grapevine (*Vitis vinifera*) from fragmented shoot apices. Vitis **17**, 335—340.

— — ; — —; 1980 a: Studies on the fragmented shoot apex of grapevine. I. The regenerative capacity of leaf primordial fragments *in vitro*. J. Exp. Bot. **31**, 483—488.

— — ; — —; 1980 b: Studies on the fragmented shoot apex of grapevine. II. Factors affecting growth and differentiation *in vitro*. J. Exp. Bot. **31**, 489—495.

— — ; — —; CLINGELEFFER, P. R.; 1981: Studies on the fragmented shoot apex of grapevine. III. A scanning electron microscope study of adventitious bud formation *in vitro*. J. Exp. Bot. **32**, 1079—1083.

BENNETT, R. K.; 1988: Studies on the development of systems for the genetic transformation of crop plants. MScAgr. Thesis, Univ. of Sydney, Australia.

FAVRE, J. M.; 1976: Influence de l'état physiologique de la plante et de la nature de l'organe prélévé sur l'obtention des néoformations caulinaires de la vigne. Congr. Nat. Soc. Savantes Lille **101**(1), 465—474.

— — ; 1977: Premiers résultats concernant l'obtention *in vitro* de néoformations caulinaires chez la vigne. Ann. Amélior. Plantes **27**, 151—169.

JAMES, D. J.; PASSEY, A. J.; RUGINI, E.; 1988: Factors affecting high frequency plant regeneration from apple leaf tissues cultured *in vitro*. J. Plant. Physiol. **132**, 148—154.

MULLINS, M. G.; SRINIVASAN, C.; 1976: Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet Sauvignon) by apomixis *in vitro*. J. Exp. Bot. **27**, 1022—1030.

NITSCH, J. P.; NITSCH, C.; 1969: Haploid plants from pollen grains. Science **163**, 85—87.

RAJASEKARAN, K.; MULLINS, M. G.; 1981: Organogenesis in internode explants of grapevines. Vitis **20**, 218—227.

REISCH, B. I.; MARTENS, M. H.; CHENG, Z. M.; 1989: High frequency shoot regeneration from grape leaves, petioles and tendrils. 5th Intern. Symp. Grape Breeding, St. Martin/Pfalz, F.R. Germany, Sept. 12—16, 1989, Abstr. 46.

SRINIVASAN, C.; MULLINS, M. G.; 1980: High-frequency somatic embryo production from unfertilized ovules of grapes. Scientia Hort. **13**, 245—252.

SRISKANDARAJAH, S.; MULLINS, M. G.; NAIR, Y.; 1982: Induction of adventitious rooting *in vitro* in difficult-to-propagate cultivars of apple. Plant Sci. Letters **24**, 1—9.

VILAPLANA, M.; MULLINS, M. G.; 1989: Regeneration of grapevines (*Vitis* spp.) *in vitro*: Formation of adventitious buds on hypocotyls and cotyledons of somatic embryos. J. Plant Physiol. **134**, 413—419.

WELANDER, M.; 1988: Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. J. Plant Physiol. **132**, 738—744.

Eingegangen am 7. 12. 1989

Dr. M. G. MULLINS
Department of Viticulture and Enology
University of California, Davis,
Davis, CA 95616
USA