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Regeneration of Grape (*Vitis labruscana* cv. Kyoho) by Shoot-Tip Culture

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We investigated the optimal levels of growth regulators, culture media, and pH on callus growth and organogenesis of in-vitro cultured 'Kyoho' grapes. Calli were induced by culturing leaf blades on an MS basal medium supplemented with 1 mg/L BA and 0.01 mg/L 2,4-D. In addition, calli originating from the exocarp and mesocarp of grape fruits developed on MS media supplemented with 0.1 mg/L IAA, NAA, or 2,4-D, or with 0.2 mg/L BA. In testing the potential for plant regeneration from shoot tips on various media, we found that the Nitsch medium, with 1 mg/L BA, was optimal for caulogenesis. The type of shoot development depended on the pH of the medium, with vigorous multiple-shoot development occurring at pH 6.0, and single shoots forming at pH 5.0. Finally, we were able to obtain rooted seedlings from the regenerated shoots that had been cultured on 1/4-strength Nitsch medium supplemented with 0.03 mg/L NAA.

Keyword: grapes, Kyoho, regeneration, shoot-tip culture

Most grape cultivars are centuries old and are more often the products of clonal selection than of breeding. In the case of wine grapes, the subtle and complex attributes of established cultivars are of utmost economic importance. Therefore, new cultivars are not sought after in regions where environmental or biotic stresses do not mandate them. Molecular genetic methods offer the possibility of making direct and specific changes in existing cultivars while otherwise preserving their integrity. Integral to most such approaches is the requirement for adventitious plant regeneration from explant tissues (Stamp et al., 1990). Adventitious shoot organogenesis has been achieved from fragmented shoot apices, internode segments, and leaves (Stamp et al., 1990).

Shoot-tip culture is used for rapid micropropagation, for overcoming some of the disadvantages of conventional propagation methods, and for producing virus-free plants. Prospects and strategies for grape micropropagation via this technique have been reported by Sasahara et al. (1981), Choi et al. (1992), and Kwon et al. (2000). The chemicals and parameters for in-vitro culture continue to be revised and optimized for several cultivars and species (Chee and Pool, 1983; Choi et al., 1992). Growth patterns in the explant greatly depend on the levels of essential nutrients and phytohormones in the culture media. This activity is modified by internal factors, e.g., the physiological age and the nutrient condition of the mother plants. Among the many exogenous factors, growth regulators are particularly important for plant differentiation and development. In addition, normal growth of the shoot depends on the composition of the media, as supplied with inorganic salts and sucrose. It is difficult to determine the optimal conditions because growth patterns vary by grape cultivar, and all the above-mentioned exogenous and endogenous factors tend to interact with each other.

Here, we report the effects of varying levels of growth regulators, inorganic salts, and pH on shoot-tip culturing of 'Kyoho' grape, the dominant cultivar in Cheonan City. In this study, we attempted to integrate our regeneration system and transformation technologies into breeding programs for the enhancement of grape germplasm quality.

MATERIALS AND METHODS

Plant Material

We used 5-cm-long shoot tips harvested in June from field-grown grapes (*Vitis labruscana* cv. Kyoho) for this study.

Callus Induction and Culture

Stems and leaves (<30 mm long) were sterilized with 70% ethanol for 10 s, then with a 2% sodium hypochlorite solution for 15 min. After being rinsed four times with sterile distilled water, they were cul-

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tured in 10-cm diameter plastic Petri dishes each containing 20 mL MS basal medium (Murashige and Skoog, 1962) supplemented with 1 mg/L of one auxin type (2,4-D, IAA, or NAA), 0.01 mg/L of a cytokinin (2iP, BA, or kinetin), and 30 g/L sucrose. The medium was solidified with 7 g/L agar, and the pH was adjusted to 5.8 prior to autoclaving. The 30 explants were sealed with Parafilm M in the Petri dishes, and were incubated at 25 ± 1°C, with a 16-h/8-h day/night photoperiod. Percent callus induction was recorded after six weeks of culture.

To examine their totipotency, we harvested fruits at the mature stage and divided them to two parts, exocarp and mesocarp. Each exocarp and mesocarp was also cultured on a B5 medium supplemented with 0.1 mg/L of an auxin (IAA, NAA, or 2,4-D) and 0.2 mg/L of a cytokinin (2iP, BA, or kinetin). Percent callus induction was recorded for the 30 explants after five weeks of culture.

Shoot-Tip Culture

Shoot tips were sterilized as described above. The explants were <1.5 mm in diameter, each comprising an apical meristem with 3 to 5 leaf primordia that were dissected from axillary buds. These tissues were cultured in 10-cm diameter plastic Petri dishes, each of which contained 20 mL of a selected type of medium supplemented with 1 mg/L 2,4-D, 0.01 mg/L BA, and 30 g/L sucrose. The tested media included: 1) Murashige and Skoog (Murashige and Skoog, 1962); 2) Nitsch (Nitsch, 1969); 3) McCown Woody plant (Lloyd and McCown, 1980); 4) Chee and Pool Vitis (Chee and Pool, 1987); 5) Gamborg (Gamborg et al., 1968); 6) Kao and Michayluk (Kao and Michayluk, 1975); 7) NLN 13 (Lichter, 1981); 8) Gresshoff and Doy (Gresshoff and Doy, 1974); and 9) Chu (Chu et al., 1975). Each medium was solidified with 3 g/L gelrite, and the pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Five shoot tips were cultured per dish, and were incubated at 25 ± 1°C with a 16 h/8 h photoperiod. Petri dishes were sealed with Parafilm M. After four weeks, we recorded the percent shooting from induced calli, noting whether the shoot pattern was multiple, single, or a combination of the two. In addition, we calculated the salt concentration of each medium and compared that with its respective ratio of shoot induction.

In separate experiments, we manipulated the pH level (5.0, 6.0, 7.0, 8.0, or 9.0) in dishes containing Nitsch media to determine its effect on patterns of explant development. This medium had been supplemented with 1 mg/L BA, 2% sucrose, and 10 mg/L $MgSO_4 \cdot 7H_2O$. To investigate the particular effects of individual growth regulators, we also treated dishes of Nitsch media with 0.1, 1, 3, or 10 mg/L of kinetin or BA. The percentages of shooting resulting from our pH and growth regulator treatments were recorded after four weeks of culture. All experiments were repeated for a total of three cycles.

Root Formation

To induce root formation, we excised and cultured shoots from our regenerants on a 1/4-strength Nitsch medium supplemented with 1 mg/L BA, 20 g/L sucrose, and 0.03 mg/L NAA. The percentage of rooting that occurred was recorded after four weeks of culture. This cycle was repeated three times.

RESULTS

Induction of Callus

Callus Formation from Leaves and Stems

We used leaf and stem explants to examine the effects of plant growth regulators in the initial callus-induction medium on the induction of subsequent adventitious embryogenesis. Each type of phytohormone produced a different response during in-vitro organ development (Table 1). For example, leaf explants cultured with NAA in the MS media showed direct root formation, whereas the same medium supplemented with 2,4-D resulted in callus induction. However, the medium with IAA gave a non-stimulative effect. Likewise, for stem explant cultures, kinetin treatments resulted in the formation of adventitious roots, while BA led to calli induction. In contrast, tissues treated with 2iP and IAA showed no initiation of calli.

Table 1. Effect of growth regulators on the percent callus induced from stem and leaf after 42 d (c, callus; r, root).

Explant	Auxin \ Cytokinin	2iP (%)	BA (%)	Kinetin (%)
Stem	2,4-D	75 (c)	100 (c)	70 (r)
	IAA	38 (r)	100 (c)	24 (r)
	NAA	67 (c)	100 (c)	67 (r)
Leaf	2,4-D	100 (c)	100 (c)	100 (c)
	IAA	24 (r)	0	0
	NAA	87 (r)	33 (r)	22 (r)

Table 2. Effect of growth regulators on percent callus induction from the mesocarp and exocarp of grape fruit after 35 d (c, callus; r, root).

Explant	Auxin \ Cytokinin	2iP (%)	BA (%)	Kinetin (%)
Mesocarp	2,4-D	0	0	0
	IAA	0	0	0
	NAA	0	22 (c)	0
Exocarp	2,4-D	100 (c)	33 (c)	0
	IAA	0	33 (c)	0
	NAA	0	100 (c)	0

Callus Formation from Exocarp and Mesocarp Tissues

Because tissues from the grape fruits did not respond well to any of our combinations of plant growth regulators in the MS medium, we replaced it with B5 media. Flesh tissues showed callus formation when treated with either BA or NAA, whereas skin tissue responded only to BA treatment (Table 2).

Shoot Induction

We attempted to induce shoot formation from the calli originating from the various grape organs (i.e., leaves, stems and fruits), but were unsuccessful regardless of the combination of growth regulators used. Therefore, the focus shifted to optimizing our shoot-tip culture technique.

The Effect of Media Type

Shoot tips were cultured on several different media supplemented with 1 mg/L 2,4-D, 0.01 mg/L BA, and 30 g/L sucrose. Almost all types tended to promote the formation of multiple shoots only, except for MS, Chu (N6), and McCown Woody. Using those media resulted in many multiple - shoots, but also some single-shoot development. The ratio of multiple- to single shoots, by media type, was: MS, 46:8; Chu (N6), 71:2; and McCown Woody, 80:7 (Fig. 1). By calculating the ion concentration for each medium, we could compare the optimal concentration of some inorganic ions for shoot formation, and identify those optimums as: 1.5 mM Ca^{+}, 0.5 mM PO_4^{-}, 0.5 mM Mg^{2+}, 10 mM NH_4^{+}, 10 mM K^{+}, and 20 mM NO_3^{-} (Fig. 2).

The Nitsch, NLN13, B5, and KM media favored only multiple-shoot development and no single-shoot formation. We also found that both the Nitsch and the NLN13 media were best for inducing multiple shoots, whereas the Nitsch medium recorded the best shoot growth pattern. Intermediate patterns of both multiple and single shoots were observed with the MS and MW media.

The Effect of Cytokinins

We used the Nitsch medium to test the effect of different concentrations of kinetin and BA on shoot

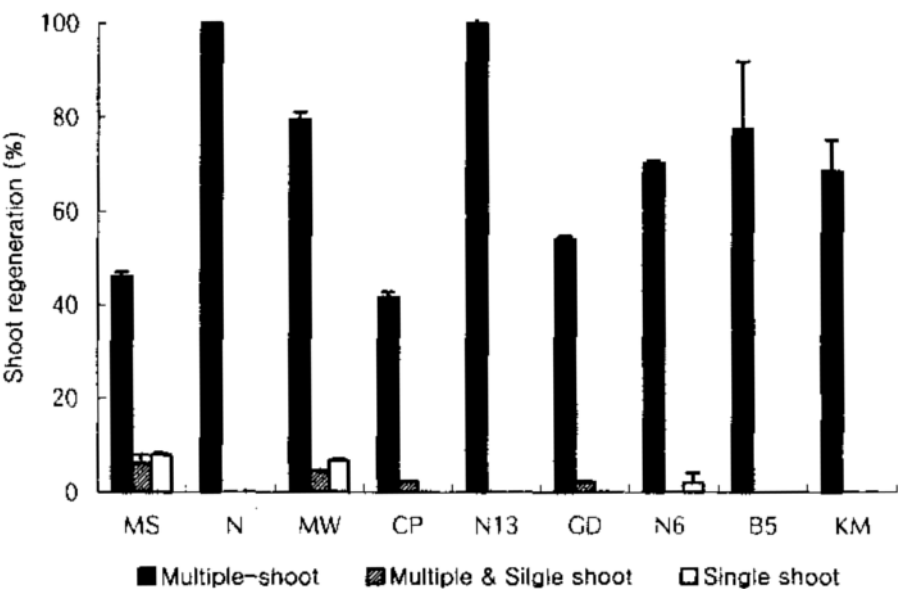

Figure 1. Percent shooting of calli from culture of *Vitis labruscana* var. 'Kyoho' in various media. Percentage was recorded after 4 weeks. MS, Murashige and Skoog; N, Nitsch; MW, McCown Woody plant; CP, Chee and Pool Vitis; N13, NLN 13; GD, Gresshoff and Doy; N6, Chu; B5, Gamborg; KW, Kao and Michayluk.

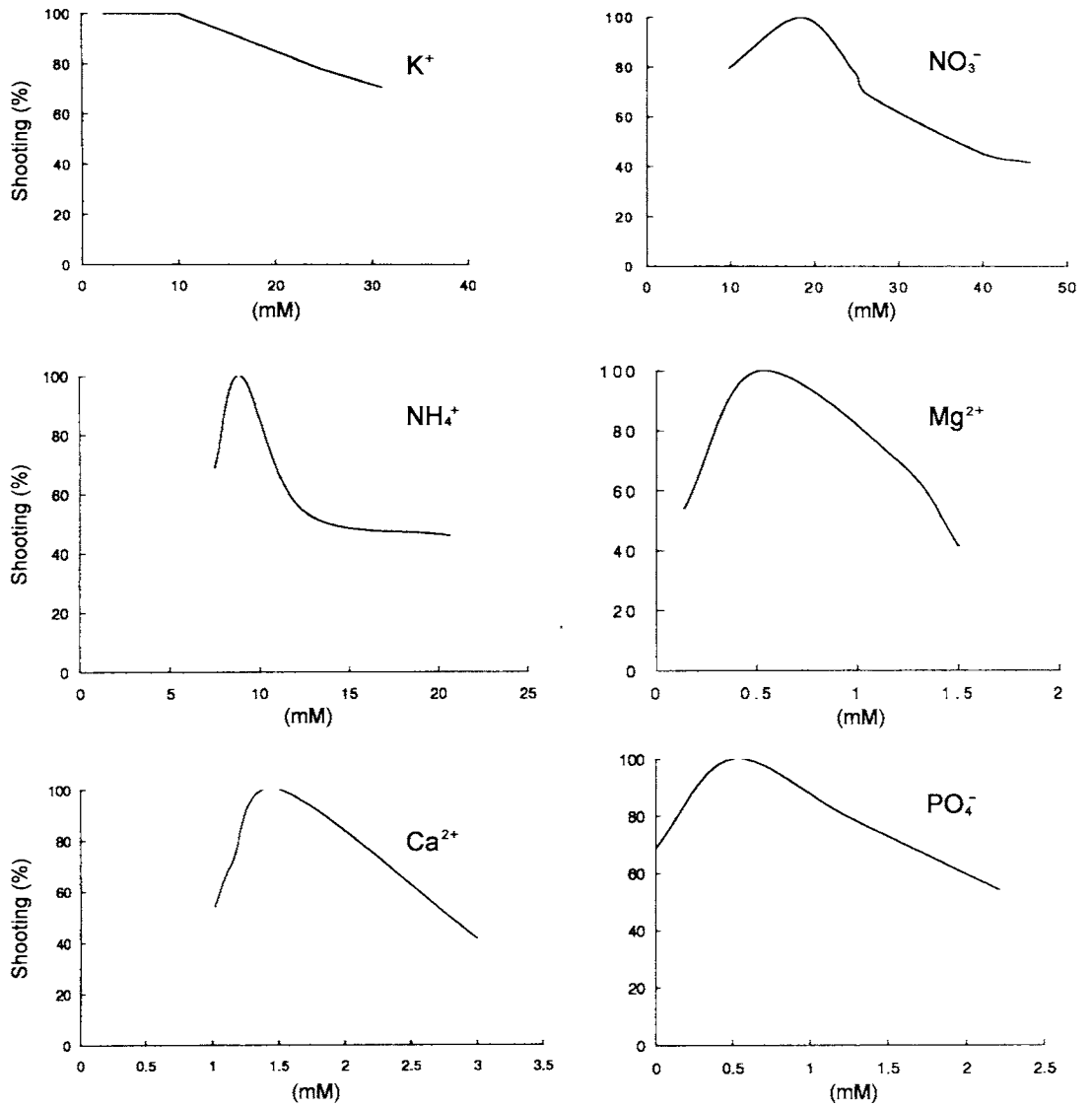

Figure 2. Effect of inorganic ions on shoot regeneration.

micropropagation (Fig. 3). Here, the multiple shoots produced on the kinetin-containing medium were stunted compared with those from media containing BA. Although the percentage of multiple shoots was high in the presence of 1 mg/L BA, not a single shoot was induced. In addition, kinetin at concentrations >1 mg/L induced single shoots, but 0.1 mg/L kinetin promoted only multiple shoots.

The Effect of pH

Explants that were grown on Nitsch media adjusted to pH 5.0 formed a high percentage of single shoots; those whose media had been adjusted to a pH level of 6.0, 7.0, 8.0, or 9.0 showed a high percentage of multiple-shoot formation, with a pH of 6.0 being optimal for promoting multiple shoots (Fig. 4).

Root Induction

We compared the in-vivo rooting response of shoots originally grown on 1/2- and 1/4-strength Nitsch media that contained either zero or 0.03 mg/L NAA. Excised shoots from these regenerants had the highest root initiation ratio and root number per shoot from the 1/4-strength medium supplemented with 0.03 mg/L

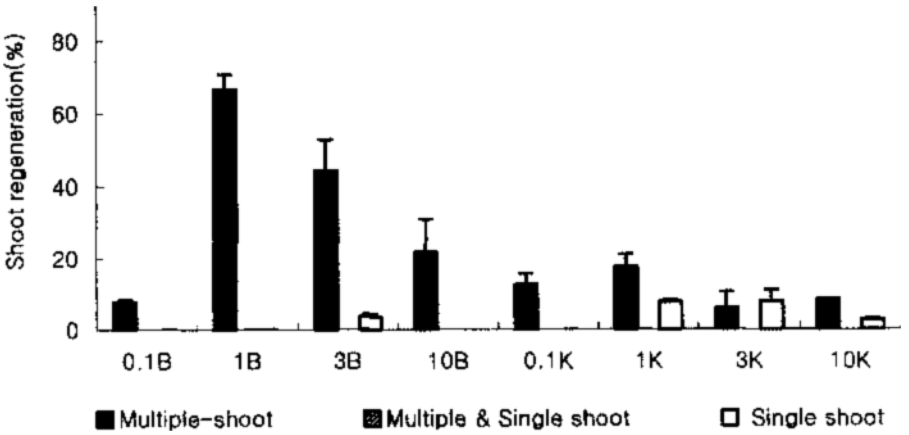

Figure 3. Effect of kinetin and BA on shooting patterns from culture of *Vitis labruscana* cv. Kyoho. Percent shooting of callus induction was recorded after 4 weeks.

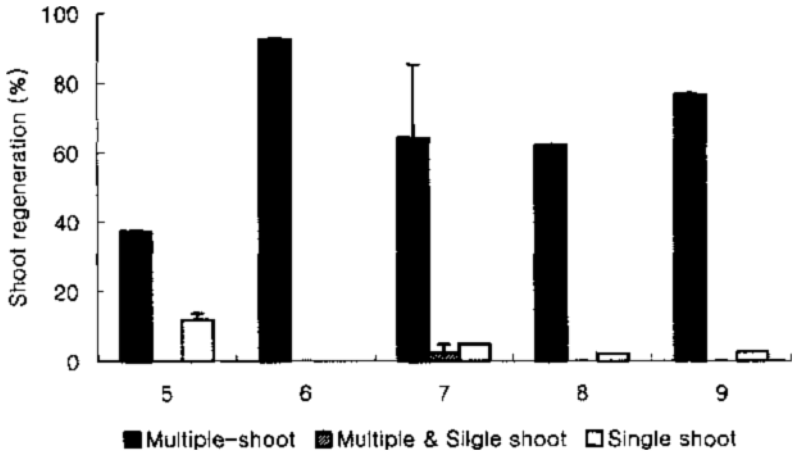

Figure 4. Effect of pH on shooting patterns from culture of *Vitis labruscana* cv. Kyoho. Percent shooting of callus induction was recorded after 4 weeks.

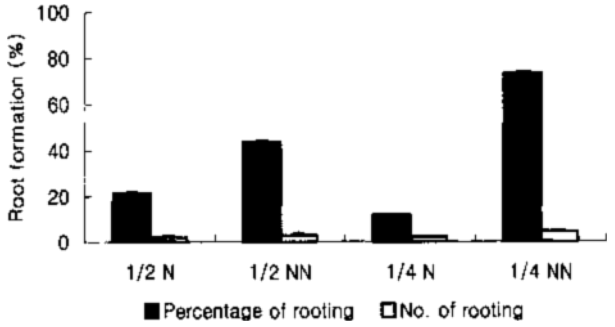

Figure 5. Effects of strength of Nitsch medium and NAA on root induction and root number. Percent shooting of callus induction was recorded after 4 weeks.

NAA. Rooting and root growth resulted from more than 70% of the adventitious shoots on this medium (Fig. 5). Multiple shoots and plantlets also originated from these cultures, as seen in Figure 6.

DISCUSSION

Because both auxins and cytokinins can affect adventitious embryogenesis from cultured grape explants (Reustle et al., 1994; Perl et al., 1995; Torregrosa et al., 1995; Nakano et al., 1997), we investigated their effects on callus induction in MS media. In our stem explant cultures, calli were induced and developed regardless of the kind of auxin present in the medium, whereas the potential for callus induction in our leaf cultures depended on a specific auxin

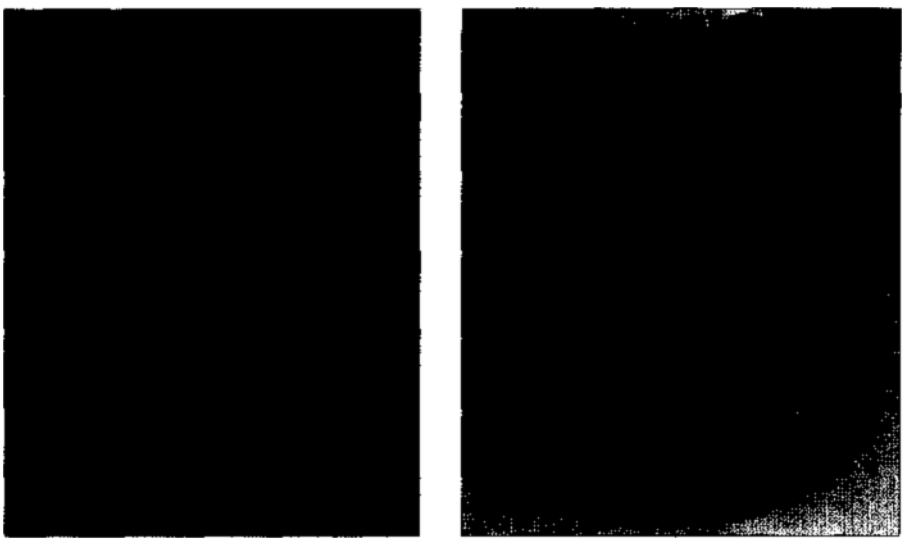

Figure 6. Plantlet originating from culture of *Vitis labruscana* cv. Kyoho (left, multiple shoots; right, 3-month-old plantlet).

type (Table 1). The nutritional requirements for callus initiation usually vary considerably for primary explants of different origin (Dodds et al., 1985). In fact, we found high frequencies from stem explant culture in response to 0.01 mg/L BA treatment, compared with optimum callus formation from leaf explants occurring with 1 mg/L 2,4-D. In addition, the two types of fruit tissue responded differently to plant growth regulators. For example, very little callus was induced from the mesocarp, whereas the exocarp formed calli in the medium treated with BA (Table 2). This suggests that primary explants having different origins also have different nutritional requirements for callus induction.

Because we were unable to induce shoot regeneration from 'Kyoho' calli, regardless of hormonal or pH treatment, we tried to establish plantlets through a regeneration system using shoot-tip culture. Several media have been proven effective for shoot multiplication in grape culture (Chee and Pool, 1985; Stamp et al., 1990; Choi et al., 1992; Emershad and Ramming, 1994; Zhu et al., 1997; Kwon et al., 2000). Therefore, we first tested their effects on shoot formation (Fig. 1). Although most of the media types tended to promote the formation of multiple- rather than single shoots, use of the MS, Chu (N6), and McCown Woody media did result in development of multiple- as well as a few single - shoots. Tissues grown on the Nitsch medium showed rapid growth and abundant multiple - shoots. Therefore, we consider the Nitsch medium to be most effective for shoot multiplication.

Ion concentrations in culture media may also affect shoot multiplication (Chi and Francois, 1991; Kwon et al., 2000; Perrin et al., 2001). For example, 9 mM NH_4^+ added to the media is more effective than 16.5 mM NH_4^+ during production of grape cultivar 'Cabernet Sauvignon' (Perrin et al., 2001). We calculated ion concentrations and determined the following optimal levels of some inorganic ions for promoting shoot formation: 1.5 mM Ca^+; 0.5 mM PO_4^-; 0.5 mM Mg^{2+}; 10 mM NH_4^+; 10 mM K^+; and 20 mM NO_3^- (Fig. 2). These results, showing that low inorganic concentrations are more effective than high concentrations, are consistent with those of Chi and Francois (1991), Kwon et al. (2000), and Perrin et al. (2001).

BA is effective for the induction of somatic embryos in grape (Stamp et al., 1990; Gray, 1992; Hyung et al., 1992; Isabelle et al., 1993). Although higher concentrations produce more shoots, the plantlets are very short and compact, and may have abnormal leaves. In contrast, lower concentrations have little influence on shoot growth (Choi et al., 1992). In the current study, we tested the effect of using different levels of kinetin and BA when culturing on the Nitsch medium (Fig. 3). The frequency for multiple-shoot production was high when the medium was supplemented with BA, whereas kinetin at concentrations >1 mg/L induced single-shoot formation. This suggests that treatment with BA is more effective in producing multiple shoots than is kinetin.

The particular pH of a nutrient medium can influence ion solubility, the ability of the agar to gel, as

well as subsequent cell growth (Torres, 1989; Zatyko and Molnar, 1990). Zatyko and Molnar (1990) found that chokeberry root formation was facilitated at a pH of 3.0. In the current study, we investigated the developmental patterns of explants as they related to pH (5.0, 6.0, 7.0, 8.0, and 9.0). We used a Nitsch medium supplemented with 1 mg/L BA, 2% sucrose, and 10 mg/L $MgSO_4 \cdot 7H_2O$. At pH 5.0, a high percentage of single shoots were formed, while a pH of 6.0 coincided with the occurrence of multiple shoots (Fig. 4). These results suggest that an acidic pH promotes single-shoot formation, and that culturing on a medium with a pH >6.0 tends to promote the development of multiple shoots.

In several studies where salt concentrations were decreased to half-strength, both the number of secondary roots and the rooting rate increased, with NAA treatments resulting in rates of >80% (Gray and Benton, 1991; Choi et al., 1992; Mozsar and Sule, 1994). In our experiments with NAA, optimal performance, as indicated by the number of roots and the rooting rate, was achieved using 1/4-strength Nitsch medium supplemented with 0.03 mg/L NAA (Fig. 5). This again demonstrates the positive effect of NAA treatments on the rooting of plantlets.

In summary, we have identified a successful shoot-tip culturing technique for improving shoot initiation and development of *V. labruscana* cv. Kyoho. The integration of our regeneration system and current transformation technologies into grape breeding programs provides an additional strategy for the enhancement of its germplasm quality.

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