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Effects of Auxins and Cytokinins on Callus Induction from Leaf Blade, Petiole, and Stem Segments of *In Vitro*-grown 'Sheridan' Grape Shoots

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Abstract

To establish an the mass production system of grape anthocyanin pigments through callus and cell suspension culture, the effects of various combinations of auxins and cytokinins on friable callus production were studied. For friable callus production, 2,4-D was superior to other regulators. IAA at 2 mg/L induced callus from stem and petiole while NAA resulted in rooting. Callus induction rate increased with the 2,4-D level, and stem segments were superior to leaf blade or petiole, showing nearly 100% with 1 and 2 mg/L 2,4-D from petiole and stem. Combined treatments of 2,4-D + kinetin and NAA + BA also yielded friable callus from stem segments. In treatments with 1 mg/L 2,4-D + 1 mg/L kinetin and 1 mg/L NAA + 1 mg/L BA, callus induction rate was nearly 100%. The combination effect of 2,4-D and BA on anthocyanin production was not significant.

Introduction

Plant cell cultures provide an attractive route to produce high-value plant-derived products, such as flavors, fragrances, alkaloids, colorants and pharmaceuticals that are very expensive to synthesize chemically and that occur naturally only at very low concentrations. With high demand of food colorants from natural sources instead of synthetic materials, anthocyanin production has been reported in different plant tissue or cell cultures (Zhang et al., 1997).

Explant source is one of the parameters for successful long-term cell culture (Krul and Mowbray, 1984). Callus culture can be done from different vegetative organs. The primary implant can be a fragment of a stem, a leaf, a petiole, a root, or a cambial tissue (Grenan, 1992). Relatively stable and fast-growing cell cultures have been isolated from young herbaceous stem explants, petioles, tendrils, and buds, and slower growing isolates were obtained from mesophyll, inflorescence, and fruit cells (Brezeanu et al., 1980).

IAA, NAA, or IBA with or without CW were utilized by early workers (Morel, 1944; Hildebrandt, 1958) for induction and continuous growth of isolated grape cells. Phenoxyacetes (2,4-D, PCPA, or NOA) are useful for callus initiation (Krul and Worley, 1977; Jona and Webb, 1978; Srinivasan and Mullins, 1980). BA is the most widely used cytokinin for the initiation and long-term growth of isolated grape cells, although kinetin (Hawker et al., 1973) or zeatin (Jona and Webb, 1978) are also effective.

The study was aimed to investigate the appropriate cytokinin-auxin combination to establish the mass production system of friable callus with the ultimate goal for the mass production of anthocyanins through the culture of anthocyanin-forming cell lines.

Materials and Methods

One-eye cuttings of 12 cm-long 'Sheridan' grape canes were rooted on sand bed in a greenhouse. Shoot tips and nodal segments were dissected from shoots after 4 weeks,

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and the explants were surface sterilized in 1% sodium hypochlorite solution containing 3 drops of Tween-20 for 15 min, and then rinsed 3 times with sterile distilled water.

Explants of ca. 0.6 cm were excised and individually transferred into test tube containing 20 mL of culture medium comprised of 1/2 Murashige and Skoog (MS) with 3% sucrose and 0.2% gelrite.

The culture conditions were maintained at 25±1℃ with a 16-hour photoperiod under 40 μmol · m^{-2} · s^{-1} PPF provided by fluorescent lamps.

Young, fully expanded leaves, petiole, and stem segments were collected from *in vitro*-grown cultures. The leaves were aseptically sliced into 1.0-cm^2 square pieces, discarding their margin. Longitudinally sectioned stems were placed to medium with cut surface down. Five explants were placed in each 9.0-cm petridish containing 20 mL of appropriate medium.

To test the effect of growth regulators, MS medium was supplemented with 2,4-D, NAA or IAA (0.01, 0.05, 0.1, 0.5, 1, and 2 mg/L), and 2,4-D or NAA (0.05, 0.1, 0.5, and 1 mg/L) + BA or kinetin (0.1, 0.5, and 1 mg/L). In all media, 8 g/L Bacto-agar (DIFCO) and 3% sucrose were used and the medium pH was adjusted to 5.8 before autoclaving at 121℃ and 1.2 kg/cm pressure for 20 min.

The explants were cultured for 6 weeks at 25±1℃ under complete darkness. Each treatment was repeated 10 times. Data were presented as mean ± SE. Statistical analyses were made using one-way analysis of variance (ANOVA).

Results and Discussion

Among tested auxins, 2,4-D was most suitable for friable callus production. Petiole and stem segments formed the callus more profusely, and calluses were formed when the concentration of 2,4-D was over 0.5 mg/L. Almost all these explants formed callus over 1.0 mg/L 2,4-D. IAA at 2 mg/L induced callus from stem and petiole segments while NAA resulted in rooting. Percent callus formation increased with 2,4-D level (Figure 1).

Full or partial co mbinations of 0.1, 0.5, and 1 mg/L BA or kinetin with either 0.05, 0.1, 0.5, and 1.0 mg/L 2,4-D or NAA were made to assess their ability to form the callus. Overall response to hormones was superior in stem segments while leaf explants resulted in poor callus induction. The combined 2,4-D and BA at high level were favorable for callus induction from stem segments. Percent callus formation of stem segment was higher than leaf blade and petiole, and decreased with the decreasing concentration of 2,4-D and BA. Most stem segments exhibited 100% callus formation in all hormonal combinations except for 0.05 mg/L 2,4-D + 0.1 mg/L BA (Figures 2-4). Especially, the combination of 1.0 mg/L 2,4-D and BA showed 100% callus induction.

Combined treatments of 2,4-D + kinetin and NAA + BA also yielded friable callus from stem segments. In treatments with 1 mg/L 2,4-D + 1 mg/L kinetin and 1.0 mg/L NAA + 1 mg/L BA, nearly 100% callus induction was observed. Callus formation decreased as the concentration of 2,4-D and kinetin decreased, and increased with the increasing concentration of 2,4-D. And the best result was

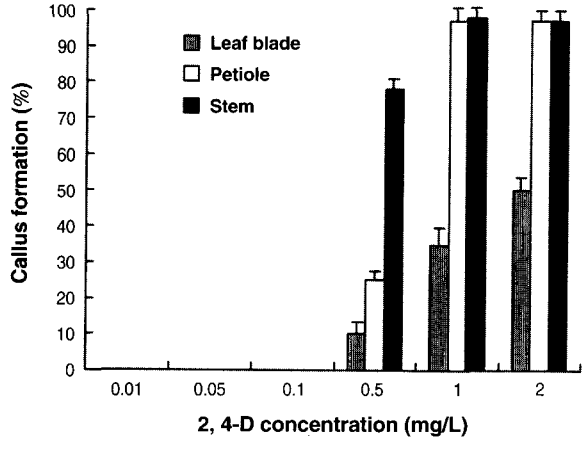

Figure 1. Effect of 2,4-D on callus formation from leaf blade, petiole, and stem segments of *in vitro*-grown grape shoots. Bars represent mean ± SE (n=10).

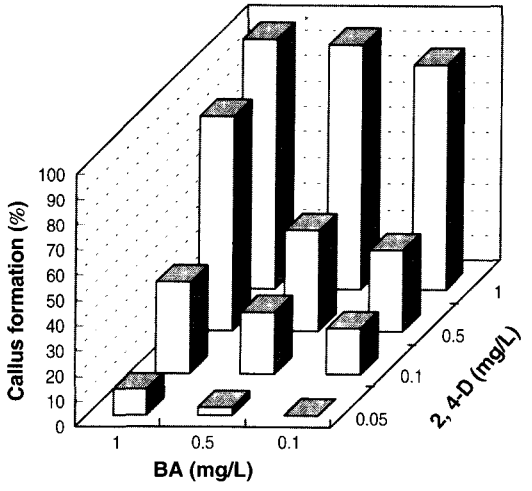

Figure 2. Callus formation from leaf blade of *in vitro*-grown grape shoots as affected by 2,4-D and BA.

obtained with stem segments showing almost 100%. There were significant differences among explant origin in combination of NAA and BA, and most of the stem segments formed callus in all combinations, except for 0.1 mg/L BA + 0.05 mg/L 2,4-D or 0.1 mg/L NAA + 0.05 mg/L BA (Figures 5-10).

In single teatment of auxins, callus was induced only with 2,4-D, and stem segments responded more favorably. Callus was induced at rather higher hormone concentration. The combined treatment of auxin and cytokinin induced callus at high concentration from stem segments. But good callus was obtained from leaf segments.

Upon subculture, callus obtained from both leaf and stem explants of either *Rosa* species readily oxidized, changing the callus color to light tan-brown. Several factors among which callus size and light condition may be responsible for the observed deterioration. Slicing the callus into small pieces (2-2.5 mm^2) increased the likelihood of oxidation. Callus originating in the dark was more sensitive than that from the light.

Lee and Wetzsteinl (1988) reported that protoplasts cultured in liquid medium adhered and formed aggregates. The culture medium turned brown and protoplasts ultimately disintegrated with no cell division. In grape protoplast survival (Shimizu, 1985), as well as cell division frequency were improved in solidified versus liquid medium. Solidified medium may provide support required for protoplast division.

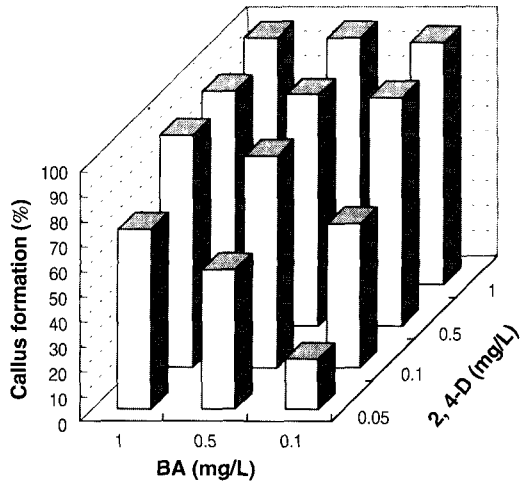

Figure 3. Callus formation from petiole of *in vitro*-grown grape shoots as affected by 2,4-D and BA.

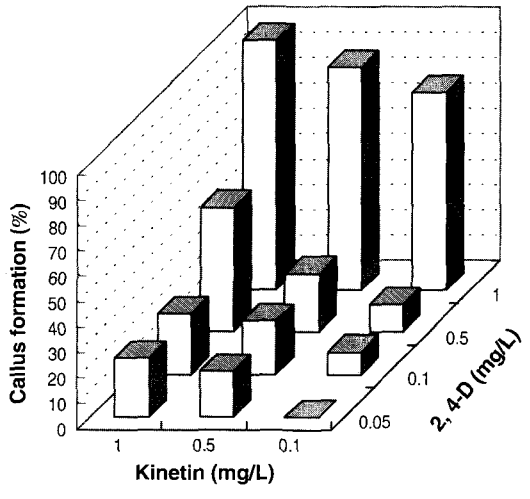

Figure 5. Callus formation from leaf blade of *in vitro*-grown grape shoots as affected by 2,4-D and kinetin.

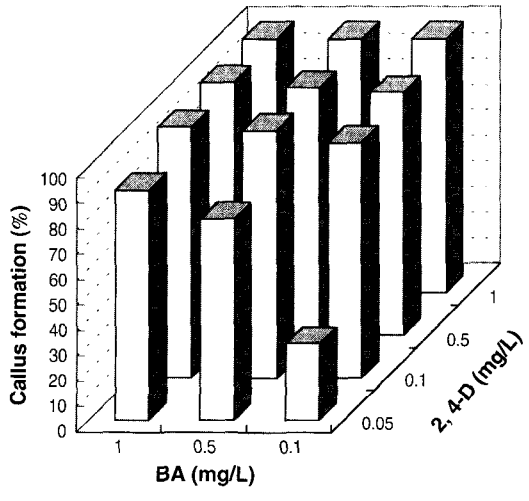

Figure 4. Callus formation from stem of *in vitro*-grown grape shoots as affected by 2,4-D and BA.

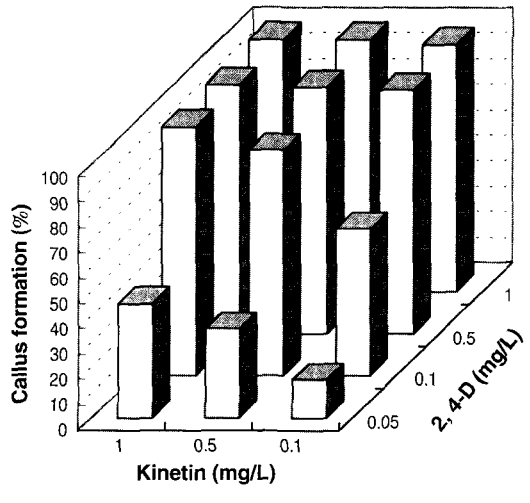

Figure 6. Callus formation from petiole of *in vitro*-grown grape shoots as affected by 2,4-D and kinetin.

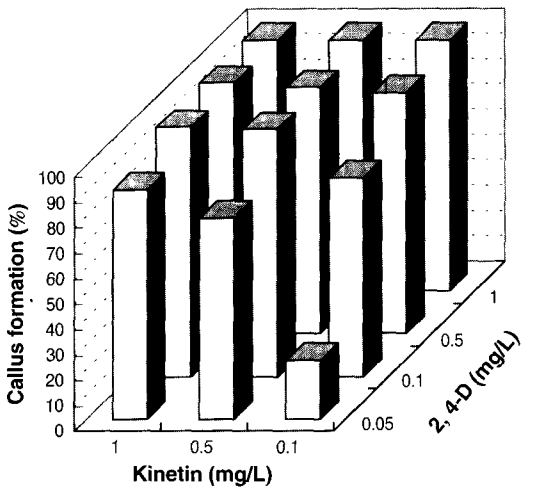

Figure 7. Callus formation from stem of *in vitro*-grown grape shoots as affected by 2,4-D and kinetin.

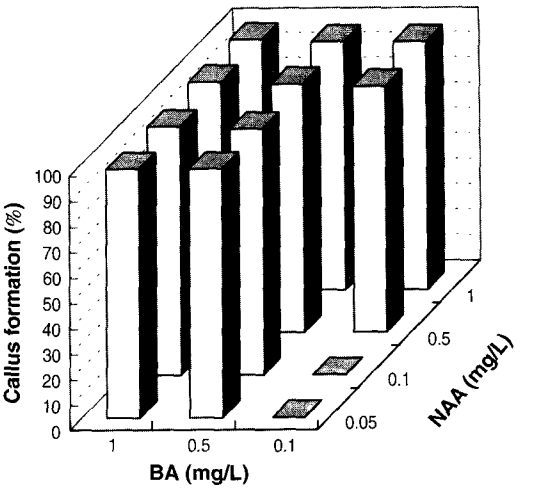

Figure 9. Callus formation from petiole of *in vitro*-grown grape shoots as affected by NAA and BA.

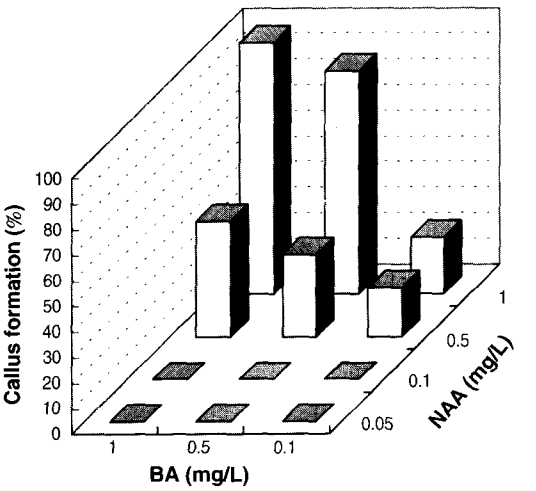

Figure 8. Callus formation from leaf blade of *in vitro*-grown grape shoots as affected by NAA and BA.

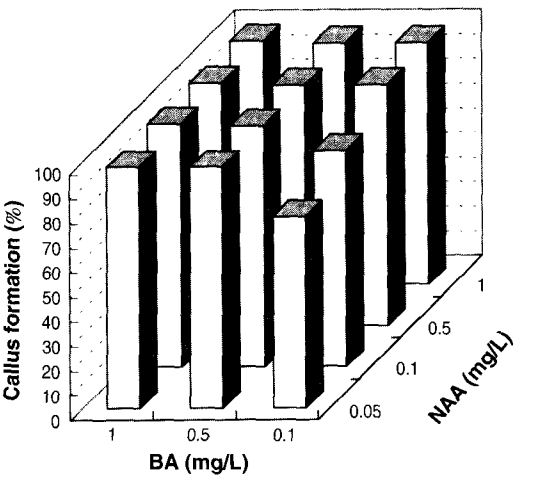

Figure 10. Callus formation from stem of *in vitro*-grown grape shoots as affected by NAA and BA.

Callus production from anthers of *Vitis latifolia* L. (wild grape) cultured on Nitsch and Nitsch medium supplemented with 20 μM 2,4-D and 9 μM BA was the highest (Salunkhe et al., 1999).

Callus was induced from tendrils of *Vitis vinifera* L. when the medium was supplemented with 10 μM BA, 0.4 μM NAA and 2.8 μM GA (Salunkhe et al., 1997). Katsirdakis and Roubelakis-Angelakis (1992) reported that the highest viability of grape leaf protoplasts was obtained with 2.3 μM BA and 10 to 15 μM NAA. Thidiazuron (TDZ) (2.3 to 4.5 μM) alone or in combination with BA (1.0 to 5.0 μM) or kinetin (1.0 or 5.0 μM) was effective for establishing callus cultures from axillary buds (Sudarsono and Goldy, 1991). Nitsch and Nitsch medium supplemented with 10.7 μM NAA and 0.9 μM BA was effective for proliferation of embryogenic callus (Robacker, 1993).

In this experiment, callus was obtained from grape cultured *in vitro* at high auxin and cytokinin level and placed in the dark. It is well established that in order to facilitate cellular proliferation, growth substances such as IAA, NAA, 2,4-D or kinetin at high concentrations are added to the medium(Grenan, 1992).

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