Response of In Vitro Strawberry to Silver Nitrate (AgNO₃)

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Abstract. Response of Toyonoka strawberry to AgNO₃ was studied. Types and combinations of plant growth regulators had significant effects on shoot regeneration efficiency. Explants cultured for 10 days in shoot regeneration medium in the presence of AgNO₃, not only enhanced shoot regeneration efficiency but also expedited the initiation of adventitious buds. Highest regeneration (87.38%) and number of shoots per explant (11.67) were achieved in shoots regeneration media containing 1.5 mg·L⁻¹ TDZ, 0.4 mg·L⁻¹ IBA and 1.0 mg·L⁻¹ AgNO₃. Half-strength MS containing 1.0 mg·L⁻¹ AgNO₃ was an optimum medium for rooting. AgNO₃ advanced root emergence and increased percent rooting, root length, dry weight and activity. Lower concentrations of AgNO₃ inhibited ethylene production and promoted shoot regeneration and growth. It had a significant stimulatory effect on chlorophyll, soluble protein contents and antioxidant enzyme activities. Chlorophyll and soluble protein contents, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were increased in the presence of AgNO₃ and reached maximum at 1.0 mg·L⁻¹ AgNO₃. Root water content, superoxide free radicals (O⁻₂⁻) and malondialdehyde (MDA) content, proline accumulation and IAA-oxidase activity in leaves were increased while (IAA) content was decreased in the presence of AgNO₃. Chemical names used: indole-3-butyric acid (IBA); silver nitrate (AgNO₃); thidiazuron (TDZ); N₆-benzyladenine (BA); 2,4-dichlorophenoxy acetic acid (2,4-D); indole-3-acetic acid (IAA); α-naphthalene acetic acid (NAA); gibberellic acid (GA₃); bovine serum albumin (BSA); 2,3,5-triphenyl-2H-tetrazolium chloride (TTC).

Shoot regeneration capability of strawberry cultivars varies greatly and experiments need to be conducted to develop an optimized protocol for individual cultivars. In this study, Fragaria ×ananassa Duch. ‘Toyoka’ was used to define its regeneration capability and physiological responses to AgNO₃ under in vitro conditions. The objectives of this study were to establish an efficient shoot regeneration system in the shortest possible time without entering callus phase, and to study the physiological effects of AgNO₃ in strawberry. This highly efficient in vitro regeneration protocol will be a valuable tool in genetic transformation and other fundamental studies of strawberry.

Materials and Methods

Plant material and media. Runner tissues of strawberry were washed with tap water for 30 min and surface disinfected in 70% (v/v) ethanol for 10 s and 0.1% (v/v) HgCl₂ for 5 to 8 min, followed by four washes with sterile distilled water. Each time tissues soaked in water for 5 min. Shoot tips of 0.2 mm were aseptically dissected out under a stereomicroscope and cultured on trigger medium for 3 to 4 weeks. Microshoots were transferred to proliferation medium and cultured for 3 weeks. Healthy and fully expanded strawberry leaves from plantlets were used in all experiments. After discarding apices, margins and midribs, 4 × 4-mm explants were placed adaxially on the medium. MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar with pH 5.8 was used throughout the course of study. Fifteen factorial combinations of TDZ (0.5, 1, 1.5, 2, 2.5 mg·L⁻¹), IBA (0.2, 0.4, 0.6, 0.8, 1.0 mg·L⁻¹) and 2,4-D (0, 0.2, 0.4, 0.6, 0.8, 1.0 mg·L⁻¹) were tested for their effects on shoot regeneration. After screening various combinations of growth regulators, media were named based on the best combinations and used in the preceding experiments (Table 1). Leaf discs were cultured on shoot regeneration medium with 0, 0.5, 1, 2, 4 mg·L⁻¹ AgNO₃ for 10 d and were transferred to the same medium without AgNO₃. For rooting, shoots (2.0 cm in length) were separated and cultured on ½-strength MS medium supplemented with 0 to 4.0 mg·L⁻¹ AgNO₃.

Growth regulators and AgNO₃ solutions were filter sterilized and added to the autoclaved medium in the aseptic conditions. Cultures were incubated at 25 °C ± 2 °C under 2000 Lx irradiance (provided by fluorescent lamp, 36-W, Philips Electronic N.V., Holland) with 16-h photoperiod.

Enzyme extraction. Fresh leaves (0.3 g) were homogenized in an ice bath containing 3 mL 50 mM potassium phosphate buffer

Table 1. Medium and combinations of plant growth regulators.

| Medium               | Combinations of plant growth regulators (mg·L⁻¹) | Explant type                      |
|----------------------|--------------------------------------------------|----------------------------------|
| Trigger medium       | 0.2 BA + 0.01 IBA + 0.1 GA₃                      | Shoot-tips                       |
| Proliferation medium | 0.5 BA + 0.03 NAA                                | In vitro micro shoot-tips        |
| Shoot regeneration   | 1.5 TDZ + 0.4 IBA                                | Leaf discs                       |
| Shoot regeneration plus AgNO₃ | 1.5 TDZ + 0.4 IBA + 1.0 AgNO₃                      | Leaf discs                       |
| Rooting medium       | Half-strength MS medium + 1.0 AgNO₃              | Shoots                           |

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standard. Chlorophyll content was determined using Popham and Novacky (1991) method. POD and CAT activities was conducted according to Giannopolitis (mg·L–1) regeneration x buds/explant. The linear regression formula is y = –2.01x + 2.27. The linear regression formula is y = –32.83x + 32.316. The polynomial regression formula is y = –78.79x² + 10.754x + 32.316. The polynomial regression formula is y = –0.34x² + 7.81. The polynomial regression formula is y = –2.01x + 2.27. The linear regression formula is y = –32.83x + 32.316. The linear regression formula is y = –2.01x + 2.27.

Table 2. Effects of TDZ on shoot regeneration of strawberry.

| TDZ concn¹ (mg·L–1) | Shoot regeneration² (%) | Adventitious buds/explant³ (no.) |
|----------------------|--------------------------|----------------------------------|
| 0.5                  | 24.55                    | 1.29                             |
| 1.0                  | 36.84                    | 1.71                             |
| 1.5                  | 45.45                    | 2.10                             |
| 2.0                  | 46.61                    | 2.58                             |
| 2.5                  | 47.45                    | 2.68                             |

¹Linear regression of shoot regeneration and linear regression of number of adventitious buds per explant on TDZ concentration was significant at α ≤ 0.05.

Table 3. Effects of various IBA concentrations with constant TDZ concentrations on strawberry shoot regeneration efficiency.

| Plant growth regulator² (mg·L–1) | Shoot regeneration² (%) | Adventitious buds/explant³ (no.) |
|----------------------------------|--------------------------|----------------------------------|
| 1.5 IBA                           | 60.34                    | 3.20                             |
| 1.0 IBA                           | 76.67                    | 5.78                             |
| 0.5 IBA                           | 74.07                    | 4.68                             |
| 0.8 IBA                           | 67.27                    | 4.49                             |
| 0.5 IBA                           | 63.64                    | 4.0                              |

¹Polynomial regression of both shoot regeneration and number of adventitious buds per explant on IBA concentration was significant at α ≤ 0.05.

Table 4. Effects of various concentrations of 2,4-D with constant TDZ and IBA concentrations on strawberry shoot regeneration efficiency.

| Plant growth regulator² (mg·L–1) | Shoot regeneration² (%) | Adventitious buds/explant³ (no.) |
|----------------------------------|--------------------------|----------------------------------|
| 1.5 IBA 2,4-D                    | 27.45                    | 1.86                             |
| 1.5 IBA 2,4-D                    | 17.54                    | 1.30                             |
| 1.0 IBA 2,4-D                    | 11.32                    | 1.17                             |
| 0.5 IBA 2,4-D                    | 6.78                     | 1.0                              |
| 1.5 IBA 2,4-D                    | 0                       | 0                                |

¹Linear regression of both shoot regeneration and number of adventitious buds per explant on 2,4-D concentration was significant at α ≤ 0.05.

Table 5. Regeneration capacity of adventitious buds of strawberry after preculturing in shoot regeneration medium with AgNO₃ for 10 d.

| AgNO₃¹ (mg·L–1) | Shoot regeneration² (%) | Adventitious buds/explant³ (no.) |
|------------------|--------------------------|----------------------------------|
| 0                | 74.32 c¹                 | 4.98 d                            |
| 0.5              | 83.95 b                  | 9.06 b                            |
| 1.0              | 87.38 a                  | 11.67 a                           |
| 2.0              | 73.61 d                  | 6.81 c                            |
| 4.0              | 39.48 e                  | 2.97 e                            |

¹Polynomial regression of both “shoot regeneration” and “number of adventitious buds per explant” on AgNO₃ concentration was significant at α ≤ 0.05. Means within a column followed by the same letters were not significantly different by SAS analysis using LSD at α ≤ 0.05.
Effects of AgNO₃ on rooting. Root primordia were observed after 5 to 6 d on rooting medium with optimum levels of AgNO₃. Rooting was advanced 1 to 3 d as of the control (Table 6). Lower levels of AgNO₃ markedly improved root length, dry weight and root activities (Fig. 2). AgNO₃ at 4.0 mg·L⁻¹ signiﬁcantly reduced root length and dry weight, however, they were higher than that of the control. At 1.0 mg·L⁻¹ AgNO₃, root length, dry weight and root activity were increased by 6.39, 6.57, and 2.13 times respectively. In contrast to the control, no signiﬁcant effect of AgNO₃ was observed on number of primary roots, root water content and rooting percentage below 1.0 mg·L⁻¹ AgNO₃. Higher concentrations of AgNO₃ inhibited rooting efﬁciency and primary roots formation. Root water content reduced progressively with increasing AgNO₃ concentration. It implied that AgNO₃ could promote the development of root ligniﬁcation.

Effects of AgNO₃ on shoot growth. Stunted growth associated with shorter petioles and smaller leaves were observed in strawberry plants grown for 21 d in rooting medium with AgNO₃. Compared with the control, no significant effect on shoot height was observed on number of primary roots, root water content and rooting percentage below 1.0 mg·L⁻¹ AgNO₃. Higher concentrations of AgNO₃ inhibited rooting efﬁciency and primary roots formation. Root water content reduced progressively with increasing AgNO₃ concentration. It implied that AgNO₃ could promote the development of root ligniﬁcation.

Effects of AgNO₃ on CAT–SOD–POD activities, O₂⁻ production rate and MDA contents. Signiﬁcant differences of antioxidant enzyme activities, O₂⁻ production rate and MDA contents were observed with the addition of AgNO₃ in the medium. It had a signiﬁcant stimula-
**Table 7.** Shoot growth of strawberry as affected by AgNO3.

| AgNO3 (mg·L⁻¹) | Shoot ht (cm) | Shoot dry wt (g) | Chlorophyll content (µg·g⁻¹ fresh wt) | Content of soluble protein (µg·g⁻¹ fresh wt) |
|----------------|---------------|------------------|---------------------------------------|---------------------------------------------|
| 0              | 3.52 a       | 0.097 a          | 4.296 b                               | 409.64 b                                    |
| 0.5            | 3.482 a      | 0.090 b          | 3.72 bc                               | 486.34 a                                    |
| 1.0            | 3.62 a       | 0.0891 b         | 4.58 a                                | 521.80 a                                    |
| 2.0            | 2.87 b       | 0.075 c          | 3.68 bc                               | 346.86 b                                    |
| 4.0            | 2.45 c       | 0.060 d          | 3.48 c                                | 254.67 b                                    |

*Means within a column followed by the same letters were not significantly different by SAS analysis using LSD at α ≤ 0.05.

**Table 8.** CAT, SOD, POD activities, O₂⁻ production rate and MDA content of strawberry as affected by AgNO3.

| AgNO3 (mg·L⁻¹) | CAT activity (Unit/g fresh wt) | SOD activity (Unit/g fresh wt) | A-POD activity (Unit/g fresh wt) | G-POD activity (Unit/g fresh wt) | O₂⁻ production rate (nmol·g⁻¹·min⁻¹) | MDA content (nmol/g fresh wt) |
|----------------|-------------------------------|-------------------------------|----------------------------------|----------------------------------|---------------------------------------|-------------------------------|
| 0              | 0.26 c                        | 290.51 c                      | 0.22 e                           | 0.74 d                           | 5.85 c                                | 4.43 d                        |
| 0.5            | 0.39 bc                       | 306.58 b                      | 0.36 c                           | 3.21 b                           | 6.32 c                                | 6.62 c                        |
| 1.0            | 0.67 a                        | 326.27 a                      | 0.68 a                           | 5.25 a                           | 6.56 c                                | 8.86 b                        |
| 2.0            | 0.54 ab                       | 294.94 c                      | 0.55 b                           | 3.40 b                           | 10.17 b                               | 9.54 b                        |
| 4.0            | 0.45 abc                      | 277.48 d                      | 0.24 d                           | 2.21 c                           | 14.71 a                               | 20.13 a                       |

*Means within a column followed by the same letters were not significantly different by SAS analysis using LSD at α ≤ 0.05.

**Table 9.** Proline accumulation, IAA content, IAA-oxidase activity and ethylene production of strawberry as affected by AgNO3.

| AgNO3 (mg·L⁻¹) | Proline content (µg·g⁻¹ fresh wt) | IAA content (µg·g⁻¹ fresh wt) | IAA-oxidase activity (µg·IAA/g (FW)/h) | Ethylene production (nl·g⁻¹·h⁻¹) |
|----------------|-----------------------------------|------------------------------|----------------------------------------|---------------------------------|
| 0              | 26.88 c                           | 1.72 a                       | 14.72 e                                | 1.07 c                          |
| 0.5            | 63.54 d                           | 1.49 b                       | 15.69 d                                | 0.72 d                          |
| 1.0            | 73.89 c                           | 1.30 c                       | 16.11 c                                | 0.55 d                          |
| 2.0            | 78.18 b                           | 0.58 d                       | 18.08 b                                | 1.77 b                          |
| 4.0            | 97.10 a                           | 0.41 e                       | 22.07 a                                | 4.72 a                          |

*Means within a column followed by the same letters were not significantly different by SAS analysis using LSD at α ≤ 0.05.

Discussion

Being a heavy ionic metal, AgNO₃ had dual effects on shoot regeneration and growth of strawberry. In this experiment, AgNO₃ at lower concentrations promoted shoot regeneration and plant growth. Lower concentrations of AgNO₃ inhibited ethylene production and promoted shoot regeneration and plant growth while higher concentrations of AgNO₃ promoted ethylene production. Ethylene disrupts auxin translocation, induces hyperhydricity, reduces chlorophyll content and causes tissue mortality (Lentini et al., 1988). It has been reported that Ag⁺ interferes with the binding of ethylene receptor site and helps reduce ethylene production with promotion of polyamine biosynthesis (Roustan et al., 1990). The main function of AgNO₃ is to eliminate the potential danger to plant cells and tissues in liverwort caused by ethylene (Elmo and Beyer, 1979).

In light of these reports, results in this study clearly indicated that specific binding of Ag⁺ to certain ethylene receptors was most likely responsible for its stimulating effect on shoot regeneration and rooting.

Root is the major organ that absorbs water and nutrients. Root activity is an indicator of growth and vigor of the plants (Jiang and Zhu, 1999). It has been reported that AgNO₃ at the appropriate concentrations enhanced in vitro faba bean root number, root growth rate and root length (Mutasim and Kazumi, 2000). In this study, it was observed that AgNO₃ at appropriate concentrations could promote rooting efficiency. Higher proline accumulation and IAA-oxidase activities had a positive relationship with AgNO₃ concentration while IAA content had a negative relationship with AgNO₃ (Table 9). Compared to the control, AgNO₃ at 0.5 and 1.0 mg·L⁻¹ AgNO₃ increased ethylene production and promoted shoot regeneration and plant growth. There was no significant effect on ethylene production at ≤1.0 mg·L⁻¹ AgNO₃ concentration while ≥2.0 mg·L⁻¹ AgNO₃ significantly promoted ethylene production.

Effects of AgNO₃ on proline accumulation, IAA content, IAA-oxidase activity and ethylene production. Significant differences in proline accumulation, IAA content and IAA-oxidase activities were observed during the growth of strawberry treated with AgNO₃. Higher proline accumulation and IAA-oxidase activities had a positive relationship with AgNO₃ concentration while IAA content had a negative relationship with AgNO₃ (Table 9). Compared to the control, AgNO₃ at 0.5 and 1.0 mg·L⁻¹ decreased ethylene production and promoted shoot regeneration and plant growth. There was no significant effect on ethylene production at ≤1.0 mg·L⁻¹ AgNO₃ concentration while ≥2.0 mg·L⁻¹ AgNO₃ significantly promoted ethylene production.

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membrane lipid peroxidation (Jiang et al., 1994). In our case, lower concentrations of AgNO₃ improved plant defense mechanism. \(O_2^\cdot\) and MDA content significantly increased while antioxidant activities decreased in the presence of higher AgNO₃ concentrations. This may be due to the accumulation of \(H_2O_2\) and high oxidation of membrane lipids.

In this experiment, IAA-oxidase activity and proline accumulation were increased with AgNO₃ while endogenous IAA content decreased. Reason for increasing rooting efficiency maybe attributed to the lower endogenous IAA content. Inverse-correlation between IAA content and IAA-oxidase activity was found in this study; which is in agreement with Jasdawala et al. (1977). IAA-oxidase is one of the key enzymes to regulate endogenous IAA level and interfere in auxin biosynthesis (Reinecke and Bandurski, 1988). Stresses induced proline accumulation in plants. Proline is involved in releasing stresses; protection of plant organogenesis has not yet been clearly understood and further research needs to be carried out.

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