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*Original Article*

# Influence of medium formula and silver nitrate on *in vitro* plant regeneration of *Zinnia* cultivars

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**Abstract**

The effects of culture medium formula and silver nitrate ($AgNO_3$) on plant regeneration were investigated *in vitro* using shoot tips of three zinnia cultivars namely *Zinnia angustifolia* cv. Starbright, *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland. Shoot organogenesis of all cultivars cultured on KS (Kohlenbach and Schmidt, 1975) medium was found superior to FK (Fukuda and Komamine, 1980), MS (Murashige and Skoog, 1962) and ½MS. Callus formation was found best on MS medium containing 0.1 µM TDZ. The addition of 2 mg/l $AgNO_3$ to KS medium resulted in improvement of the regeneration frequency in *Zinnia angustifolia* cv. Starbright but not in *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland. In contrast, callus formation was reduced in all three zinnias. These results indicate that the response to $AgNO_3$ was genotype dependent.

**Key words:** organogenesis, regeneration, silver nitrate, tissue culture, zinnia

## 1. Introduction

Zinnia is a cosmopolitan ornamental plant and belongs to the Asteraceae. Zinnias are propagated by seeds hence genetic variation and a tendency for stock plants to decline in vigor over time is high (Stieve *et al.*, 1992). An *in vitro* culture technique for zinnia offers the ability to uniformly mass-produce elite lines and a long-term maintenance of valuable germplasm developed through breeding programs. Recently reports on using tissue culture technique for zinnias have significantly increased (Church and Galston, 1988; Rogers *et al.*, 1992; Pesquet *et al.*, 2005). However, information concerning details of medium formula and growth regulator amendments is still a fundamental requirement of the certain Zinnia cultivars. It is generally known that tissue and callus culture induce ethylene biosynthetic pathway resulting in growth inhibition. Silver nitrate which is an inhibitor of ethylene action was reported to act as a stimulant and inhibitor in several plant tissue cultures (Al-Khayri and Al-Bahrany, 2001). In this context, the present communication describes the influence of culture medium and silver nitrate in three zinnia cultivars.

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## 2. Materials and Methods

### 2.1 Explant preparation

The zinnia seeds used in this study were purchased from the AFM Flower Seed Co., Ltd., Thailand. Three cultivars were investigated including *Zinnia angustifolia* cv. Starbright, *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland. These seeds were first washed with distilled water and surfaced sterilized with 70% ethanol for 30 second then surface sterilized using 20% (v/v) Clorox™ (6% sodium hypochlorite, active ingredient) for 20 min with 2 drops of Tween-20 per 100 ml of 20% Clorox followed by 10% Clorox™ for 20 min, respectively. The sterilized seeds were double rinsed with sterile distilled water to remove traces of the disinfectant. The seeds were sown on solid MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and without growth regulators.

### 2.2 Plant regeneration and callus proliferation

The emerged shoots (1 cm long) were capitated transversely and transfer red to 4 different shoot multiplication media, namely KS (Kohlenbach and Schmidt, 1975), FK (Fukuda and Komamine, 1980), MS, and ½MS media (Table 1) supplemented with 4.44 µM BA and 2.46 µM IBA. After 4 weeks of culture, the numbers of regenerated shoots and roots and callus formation were recorded. For callus induction, MS media supplemented with TDZ, 2, 4-D, IBA and NAA at the concentration of 0.1-1 µM were tried. To test the effect of $AgNO_3$ on plant regeneration, regenerated shoots taken at the end of an 8-week maintenance stage were transferred to KS medium containing several concentrations of 1, 2, 4, 8, and 16 mg/l $AgNO_3$.

### 2.3 Media preparation and culture conditions

All media were solidified with 0.7% Mermaid™ commercial agar. The pH of all media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm$^2$, 121°C for 20 min. All cultures were incubated at 25±1°C air temperatures in a culture room with 16 hr photoperiod under cool-white fluorescent light of approximately 3000 lx. Plant materials were stored in 115 ml glass-capped culture jars each containing 20 ml of medium.

### 2.4 Data analysis

One explant was implanted per culture and 20 cultures were raised for each treatment. All experiments were conducted on three different dates. Shoot numbers and length were submitted to analyze the variance and mean of number and length were compared using Sheffe's test at $p \leq 0.05$. The software used was SPSS version 10.0.1 Package for Windows XP Professional.

## 3. Results

Four different culture media were examined for the formation of shoot number and shoot length from cultured shoot segments of 3 zinnia cultivars. In *Z. angustifolia* cv. Starbright, a maximum of shoot number and length was found on FK and KS media but did not differ significantly. Mean shoot number and length was 4.49 and 1.52 cm ($p \leq 0.05$) on FK medium while mean shoot number and length was 4.73 and 1.85 cm ($p \leq 0.05$) on KS medium after cultured for 4 weeks (Table 2, Figure 1a). However, mean shoot number of *Z. angustifolia* cv. Starbright cultured on KS media was signi-

**Table 1.** Formulations of four culture media used for plant regeneration in three zinnia cultivars (µM)

| | KS medium (Kohlenbach & Schmidt, 1975) | FK medium (Fukuda & Komamine,1980) | MS medium (Murashige & Skoog, 1962) | ½ MS medium |
|---|---|---|---|---|
| $KNO_3$ | 9400 | 20000 | 18790 | 9395 |
| $NH_4NO_3$ | 9000 | - | 20612 | 10306 |
| $NH_4Cl$ | - | 1000 | - | - |
| $MgSO_4$ | 750 | 1000 | 2189 | 1095 |
| $CaCl_2$ | 1500 | 1000 | 2992 | 1446 |
| $KH_2PO_4$ | 500 | 500 | 1222 | 611 |
| $MnSO_4$ | 100 | 100 | 131 | 60.5 |
| $H_3BO_3$ | 160 | 160 | 100 | 50 |
| $ZnSO_4$ | 35 | 35 | 30 | 15 |
| $NaMoO_4$ | 1 | 1 | 1 | 0.5 |
| $CuSO_4$ | 0.1 | 0.1 | 0.1 | 0.05 |
| $Na_2EDTA$ | 100 | 100 | 100 | 50 |
| $FeSO_4$ | 100 | 100 | 100 | 50 |
| Glycine | 27 | 27 | 27 | 13.5 |
| Myo-inositol | 550 | 550 | 925 | 462.5 |
| Nicotinic acid | 41 | 41 | 4.06 | 2.03 |
| Pyridoxine HCl | 2.4 | 2.4 | 0.50 | 0.25 |
| Thiamine HCl | 1.5 | 1.5 | 0.3 | 0.15 |
| Folic acid | 0.11 | 0.11 | - | - |
| Biotin | 0.2 | 0.2 | - | - |
| Adenine $SO_4$ | 150 | - | - | - |
| Glutamine | 100 | - | - | - |
| Sucrose (g/l) | 10 | 10 | 30 | 15 |

**Table 2**. Effect of media formula on shoot and root formation in three zinnia cultivars after 4 weeks in culture

| Zinnia cultivars | Types of media | No. of shoots (mean ? SE ) | Shoot length (cm) | Root ranking (%) | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 |
| *Z. angustifolia* cv. Starbright | MS | 1.96 ± 1.80[b] | 1.35 ± 0.94[b] | 0 | 0 | 2.0 |
| | ½MS | 1.72 ± 1.45[b] | 1.20 ± 0.78[b] | 0 | 2.0 | 0 |
| | FK | 4.49 ± 3.07[a] | 1.52 ± 0.56[ab] | 6.8 | 1.7 | 0 |
| | KS | 4.73 ± 2.93[a] | 1.85 ± 1.03[a] | 3.6 | 0 | 0 |
| *Z. haageana* cv. Percient Carpet | MS | 2.00 ± 0.93[c] | 3.54 ± 1.99[c] | 9.1 | 40.9 | 9.1 |
| | ½MS | 1.73 ± 1.42[c] | 3.21 ± 1.95[c] | 4.8 | 52.4 | 4.8 |
| | FK | 3.15 ± 0.62[c] | 2.79 ± 1.36[c] | 14.8 | 0 | 0 |
| | KS | 3.33 ± 0.94[c] | 2.66 ± 1.34[c] | 20.0 | 4.0 | 0 |
| *Z. elegans* cv. Dreamland | MS | 1.83 ± 1.34[d] | 5.63 ± 1.68[d] | 0 | 21.7 | 17.4 |
| | ½MS | 1.66 ± 0.86[d] | 1.27 ± 0.50[d] | 12.5 | 15.6 | 12.5 |
| | FK | 1.50 ± 0.83[d] | 1.10 ± 0.29[d] | 4.2 | 12.5 | 0 |
| | KS | 2.13 ± 1.14[d] | 1.50 ± 0.81[d] | 20.0 | 6.7 | 3.3 |

The different letters within column show significant difference of shoot number and length (mean ± SE) analyzed by Sheffe's test at $p \leq 0.05$.

For root formation, Ranking 1= main root shorter than 2 cm
Ranking 2= main root longer that 2-5 cm with few hairy roots
Ranking 3= main root longer than 5 cm with many hairy roots

ficantly different ($p \leq 0.05$) when compared to mean shoot number cultured on MS and ½MS media. In *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland, all media tested did not affect the mean shoot number and length significantly (Table 2, Figure 1b, c). From these results, KS was chosen for further experiments. All regenerated shoots which obtained from shoot experiments developed long, numerous roots *per se* hence no auxins were incorporated to these media. Nevertheless root regeneration in *Z. augustifolia* cv. Starbright was much poorer than the other two genotypes.

Among the growth regulators tested, MS medium fortified with 0.1 µM TDZ was best for callus induction. The percentage of callus formation was nearly 100% in three zinnia cultivars (data not shown). The texture of callus was friable and white or pale green in color. Green spots were formed on the callus and developed further (Figure 2a). Calli from three cultivars remained active after 4-8 weeks since these calluses underwent shoot and root development (Figure 2b, c). Lower concentration of TDZ (0.1 µM) stimulated

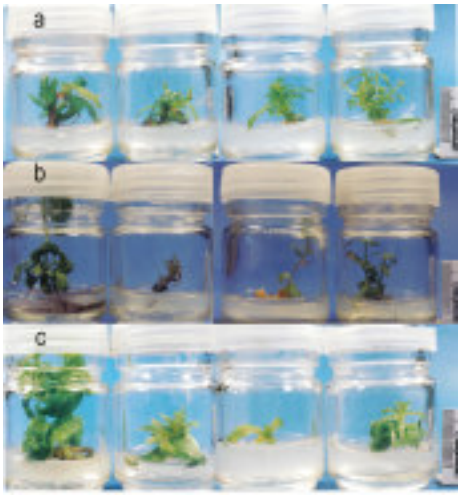


**Figure 1.** Three zinnia cultivars cultured on MS, ½ MS, FK and KS media from left to right for 4 weeks (a) *Z. angustifolia* cv. Starbright (b) *Z. haageana* cv. Percient Carpet and (c) *Z. elegans* cv. Dreamland.

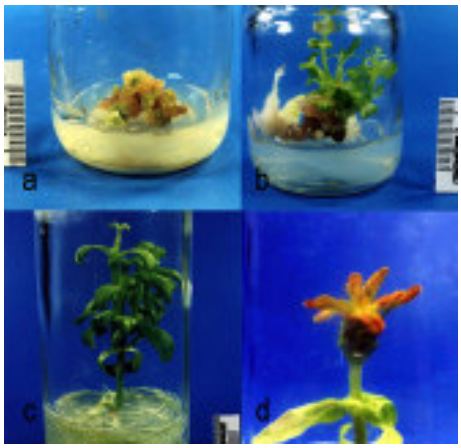


**Figure 2.** Organogenesis in *Z. elegans* cv. Dreamland (a) friable callus cultured on MS medium supplemented with 0.1 µM TDZ (b) Shoot regeneration from callus. (c) Complete plantlet and (d) *In vitro* flowering of callus derived plant.

the formation of shoots which elongated while the higher concentration (1 µM) had fewer shoots which were slow to elongate. It is interesting to note that the long-term culture of these calli could regenerate plants produced *in vitro* flower (Figure 2d).

Of the three cultivars, *Z. angustifolia* cv. Starbright produced the highest number of shoots (5.58) at the concentration of 2 mg/l $AgNO_3$ (Table 3). No statistically significant difference in the number of shoot regeneration between *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland was found. It seemed that $AgNO_3$ inhibited shoot growth of *Z. angustifolia* cv. Starbright and *Z. elegans* cv. Dreamland whilst enhanced shoot growth of *Z. haageana* cv. Percient Carpet at the concentration of 2 mg/l ($p \leq 0.05$) (Table 3). In the presence of $AgNO_3$, the best root formation was observed in *Z. haageana* cv. Percient carpet compared to *Z. angustifolia* cv. Starbright and *Z. elegans* cv. Dreamland (Table 3). In contrast, callus induction was reduced both in terms of frequency and formation in all three zinnia cultivars (data not shown).

## 4. Discussion

KS medium was very effective in this study since number of shoots and shoot length were the best for regeneration in the three zinnia cultivars especially for *Z. angustifolia* cv. Starbright. The influence of the absolute and relative amounts of nitrate and ammonium on the induction and differentiation of plant cell cultures has been reported for a number of *in vitro* systems (Grimes and Hodges, 1990; Cousson and Tran Tan Van, 1993). Considering the variable, KS medium has less concentration of nitrate and ammonium ion than the rest of the media. Therefore it might be that the three zinnia cultivars are sensitive to nitrate and ammonium ion in the culture medium. Furthermore, the presence of adenine sulphate and glutamine in KS medium may result in better axillary shoot production. This finding was in concurrence with Laliberte *et al.* (1985) who reported that adenine sulphate and tyrosine showed positive effect in *Gerbera jamesonii*.

TDZ strongly enhanced callus formation in the three zinnia cultivars as it does in other species such as *Z. marylandica* (Stieve *et al.*, 1992). Research on *Z. marylandica* showed that adventitious shoots could be derived from either 0.2 or 22.2 µM TDZ. The TDZ concentration also significantly affects the shoot regeneration in the three cultivars of zinnia. TDZ has been used to induce shoot organogenesis in callus culture of carnation (Nakano *et al.*, 1994), roses (Hsia and Korban, 1996) and orchid (Chen *et al.*, 1999). Furthermore, high activities of TDZ in plant regeneration were also reported in watermelon (Compton and Gray, 1993) and woody species (Huetteman and Preece, 1993).

The addition of $AgNO_3$ to the medium improved the regeneration frequency and reduced callus formation in all tested cultivars. Many reports have shown the positive effect of $AgNO_3$ on plant tissue culture e.g. cucumber (Mohiuddin

**Table 3** Effect of $AgNO_3$ incorporated in KS medium on shoot number, height and root formation in three zinnia cultivars after 4 weeks in culture

| Zinnia cultivars | $AgNO_3$ (mg/l) | No. of shoots (mean ? SE) | Shoot length (cm) | Root ranking (%) | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 |
| *Z. angustifolia* cv. Starbright | 1 | $2.57 \pm 1.34^{a}$ | $1.87 \pm 0.87^{c}$ | 7.1 | 0 | 0 |
| | 2 | $5.58 \pm 2.61^{b}$ | $1.71 \pm 0.27^{c}$ | 0 | 0 | 0 |
| | 4 | $3.15 \pm 2.36^{ab}$ | $1.63 \pm 0.55^{c}$ | 8.8 | 0 | 2.9 |
| | 8 | $3.27 \pm 2.57^{ab}$ | $1.71 \pm 0.54^{c}$ | 10.0 | 0 | 0 |
| | 16 | $3.59 \pm 2.29^{ab}$ | $1.32 \pm 0.48^{c}$ | 0 | 0 | 0 |
| *Z. haageana* cv. Percient Carpet | 1 | $1.92 \pm 1.08^{c}$ | $3.33 \pm 1.30^{a}$ | 16.7 | 0 | 0 |
| | 2 | $1.75 \pm 0.71^{c}$ | $7.06 \pm 2.81^{b}$ | 50.0 | 0 | 0 |
| | 4 | $2.27 \pm 1.74^{c}$ | $5.28 \pm 2.92^{ab}$ | 36.4 | 9.1 | 0 |
| | 8 | $2.08 \pm 0.76^{c}$ | $4.76 \pm 2.27^{ab}$ | 69.2 | 7.7 | 0 |
| | 16 | $2.25 \pm 1.04^{c}$ | $3.88 \pm 2.04^{ab}$ | 37.5 | 12.5 | 12.5 |
| *Z. elegans* cv. Dreamland | 1 | $2.19 \pm 1.25^{d}$ | $1.73 \pm 1.08^{d}$ | 14.3 | 14.3 | 42.9 |
| | 2 | $1.88 \pm 1.11^{d}$ | $1.45 \pm 0.64^{d}$ | 0 | 0 | 0 |
| | 4 | $1.90 \pm 1.21^{d}$ | $1.70 \pm 0.53^{d}$ | 3.3 | 10.0 | 6.7 |
| | 8 | $1.62 \pm 0.78^{d}$ | $1.43 \pm 0.45^{d}$ | 11.8 | 2.9 | 23.5 |
| | 16 | $1.47 \pm 0.83^{d}$ | $1.70 \pm 0.65^{d}$ | 0 | 0 | 0 |

The different letters within column show significant difference of shoot number and length (mean ± SE) analyzed by Sheffe's test at $p \leq 0.05$.

For root formation, Ranking 1= main root shorter than 2 cm

Ranking 2= main root longer that 2-5 cm with few hairy roots

Ranking 3= main root longer than 5 cm with many hairy roots

*et al.*, 1997); apple (Ma *et al.*, 1998); Chinese cabbage (Zhang *et al.*, 1998); cassava (Zhang *et al.*, 2001); pearl millet and sorghum (Oldach *et al.*, 2001); date palm (Al-Khayri and Al-Bahrany, 2001) and rapeseed (Akasaka-Kennedy, 2005). $AgNO_3$ at the concentration of 2 mg/l was very beneficial to shoot regeneration in *Z. angustifolia* cv. Starbright while this concentration had no effect on *Z. haageana* cv. Percient Carpet and *Z. elegans* cv. Dreamland suggested that both the response to and the optimum concentration of $AgNO_3$ were cultivar or genotype dependent. The genotype and the developmental stage of explants affected the response to $AgNO_3$ in other plant species (Zhang *et al.*, 2001). The positive effect of $AgNO_3$ at 2 mg/l in *Z. angustifolia* cv. Starbright is consistent with those reported in Chinese cabbage (Zhang *et al.*, 1998) and cassava (Zhang *et al.*, 2001). $AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot organogenesis *in vitro*. Zhang *et al.* (1998) considered that the increased of shoot regeneration frequency by $AgNO_3$ is caused by the interruption of an ethylene signal transduction pathway. Adding $AgNO_3$ to the medium resulted in shoot regeneration without the callus phase indicating that these two physiological processes are inversely correlated. In barley (Castillo *et al.*, 1998) and pea (Madsen *et al.*, 1998), the use of $AgNO_3$ has reduced rooting of regenerated shoot but in the three zinnia cultivars no adverse effect on root formation was observed.

In conclusion, the present study shows that an efficient plant regeneration system from shoot tips has been developed in the three zinnia cultivars. $AgNO_3$ influences shoot organogenesis and its efficiency is cultivar dependent.

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