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Enhancement of Adventitious Shoot Regeneration in *Cucumis sativus* L. using $AgNO_3$

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Abstract

The association of high frequencies of shoot regeneration with different concentrations of silver nitrate ($AgNO_3$) added to both seed germination and regeneration media was investigated using proximal and distal cotyledons and hypocotyls of two cucumber (*Cucumis sativus* L.) cultivars, namely Spring Swallow and Tasty Green. Addition of $AgNO_3$ to both seed germination and regeneration media had significant effect on high frequencies of shoot regeneration. The number of shoots induced per explant was also increased when $AgNO_3$ added to both media. Its presence to both media up to 50 µM further showed significant effect on shoot regeneration from non-responsive explant, distal cotyledon, of both cultivars. The best regeneration response in relation to the optimum concentration of $AgNO_3$ added to both media was found to be explant and cultivar dependent. The higher concentration negatively affected the regeneration capability as well as number of shoots induced per explant to some extent and remains unresponsive to distal cotyledon explant. Thus addition of $AgNO_3$ to both seed germination and regeneration media up to a specific concentration is essential for enhancement of shoot regeneration from recalcitrant cucumber varieties.

Introduction

Cucumber (*Cucumis sativus* L.), one of the most economically important cucurbit crops, is commercially represented by both pickling and fresh market cultivars all over the world. This species is extremely difficult to propagate vegetatively in *in vivo* condition, and therefore, the development of *in vitro* micropropagation methods (regeneration system) would be very useful for its clonal multiplication. Moreover, the development of a rapid and efficient regenerating tissue culture system is a prerequisite for the introduction of novel genes into cucumber plants

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via genetic engineering which is less time consuming and also genetically more stable and is preferred for commercial propagation. On the other hand, conventional breeding is limited because of interspecific incompatibility of this species to other species in the genus *Cucumis* except the closely related *C. sativus* var. *hardwickii* (Royle) Alef for crop improvement (Deakin et al. 1971), hence *in vitro* regeneration could be used as a competent and alternative tool.

Ethylene (C_2H_4), a gaseous plant hormone, which is produced by almost all plants, mediates a range of different plant responses and developmental steps (Abeles et al. 1992). It plays an important role in seed germination, tissue differentiation, the formation of shoot and root primordial (Abeles et al. 1992) and also seems to be involved with the poor regeneration potential or recalcitrant behavior of cultured materials (Chi and Pua 1989). In members of Brassicaceae, ethylene produced by explants in *in vitro* culture conditions was responsible for the recalcitrancy in regeneration, reported in cauliflower (Bhalla and Smith 1998) and Chinese cabbage (Zhang et al. 2000). The use of ethylene inhibitor, silver nitrate, in the shoot regeneration medium has been shown to be effective in overcoming the recalcitrance problem (Chi and Pua 1989) or significantly enhancing the regeneration response in chili (Hyde and Phillips 1996), Brassica (Eapen and George 1997; Hu et al. 1999); cassava (Zhang et al. 2001), radish (Curtis 2004) and somatic embryo formation from coffee (Fuentes et al. 2000) and anther culture of cabbage (Achar 2002). It has also been used successfully in several monocotyledonous species (rice, maize) to enhance embryogenic callus initiation and plant regeneration (Adkins et al. 1993; Vain et al. 1989).

In this paper, we first report the simultaneous addition of ethylene inhibitor, $AgNO_3$, to both seed germination and shoot regeneration media to obtain high frequency plant regeneration as well as an increase in number of shoots through direct shoot regeneration from cotyledon and hypocotyl explants of two poorly regenerative cucumber cultivars e.g. Spring Swallow and Tasty Green.

Materials and Methods

Mature seeds of cucumber (*Cucumis sativus* L.) cultivars (Sakata Seed Crop, Japan), Spring Swallow (SS) and Tasty Green (TG), used in this study were de-coated manually. The seeds with endotesta were then treated with 70% ethanol for one min and surface sterilized in a solution of 20% (v/v) clorox (commercial bleach containing 5.25% sodium hypochlorite) with one drop of Tween 20 for 15 min, followed by three rinses with sterile water. The seeds were germinated on filter paper in Petri dishes (90 mm), moistened with liquid MS. This medium is referred as seed germination medium (SGM) and four concentrations of $AgNO_3$ (10, 30, 50 and 100 μM) were added to the SGM.

Proximal cotyledon and hypocotyl, distal cotyledon and hypocotyl explants of both cucumber cultivars were obtained from eight-day-old seedlings germinated on medium containing $AgNO_3$ (SGM + $AgNO_3$). Each cotyledon was cut into four pieces by cutting longitudinally followed by transversely. The hypocotyls were bisected into two equal long (4 - 5 mm) pieces. Regeneration medium (RM) consisted of full strength MS basal nutrients, MS vitamins, 3% sucrose, 2 g/l (w/v) phytagel, different combinations of BAP (0.7, 1.0,2.0 and 3.0 mg/l) was used for *in vitro* tissue culture. $AgNO_3$ at the concentration of 10, 30, 50, and 100 μM was added separately to RM with each concentration of BAP listed above (RM + $AgNO_3$). The RM was solidified by 2 g/l gelrite (Sigma, USA) and added to the medium before autoclaving. All the media combinations were adjusted to pH 5.7 before autoclaving at 121°C for 15 min at 15 Ib/cm^2 pressures. The autoclaved regeneration medium was allowed to cool down to approximately 60°C and then dispensed into the sterile plastic Petri dishes (90 mm), approximately 40 ml per dish.

Twenty four replicates of proximal and distal cotyledons and 18 replicates of proximal and distal hypocotyl explants obtained from plants germinated in SOM containing $AgNO_3$, were cultured onto each medium formulation (RM) with $AgNO_3$ at all concentrations separately, mentioned above (TE 3, Table 1). A separate experiment was carried out with the same explants, obtained from plants germinated in SOM containing $AgNO_3$ that cultured onto regeneration medium without $AgNO_3$ (TE 2, Table 1) A control experiment was also conducted to compare the regeneration efficiency where $AgNO_3$ was not added to either seed germination or regeneration medium (TE I, Table 1). The cotyledon pieces were placed adaxially on the medium. The explants were incubated at 24 ± 1°C under fluorescent and incandescent lamps (39.3 $\mu mol.m^{-2}.S^{-1}$) for 16h per day. After three weeks, the number of shoots arising from the explants was recorded. Statistical analysis was performed by SAS and means were compared using DMRT.

Regenerated shoots were separated individually and transferred on to MS containing 0.5 mg/l IBA for elongation and rooting. Subsequently, well developed and extensively rooted plantlets were acclimatized to ambient humidity levels and allowed to grow under natural lighting and environmental conditions.

Results and Discussion

The proximal and distal cotyledon and proximal hypocotyl explants of both cultivars used in the experiments listed in the Table 1 (TE 1, 2 and 3), enlarged and induced compact and greenish calli. The higher concentration of $AgNO_3$ (100 μM) added to both SGM and RM, produced less greenish and compact calli

compared to 10, 30 and 50 μM. The amount of greenish and compact calli initiation varied depending on the explant type and concentration of $AgNO_3$ used. The induced greenish and compact calli produced green shoot primordial in 12 days, which subsequently formed organized shoot structure in 16 days of culture.

Table 1. Type of experiments carried out with $AgNO_3$, added to either SGM or both SGM and RM.

Type of experiments (TE)	Addition of $AgNO_3$	
	SGM	RM
TE 1 (control)	Not added	Not added
TE 2	Added	Not added
TE 3	Added	Added

In control experiment (TE 1), without $AgNO_3$, the proximal cotyledon and hypocotyl explants of both cultivars showed low frequency of shoot regeneration at different concentrations of BAP (Fig. 1A,C,D and F). Compared to the control, a slight improvement in shoot regeneration was observed from both explants of SS and TG cultivars when $AgNO_3$ added to the SGM only at the concentrations of 10, 30, and 50 μM (TE 2). Significant difference was not observed in shoot regeneration between control and regeneration treatment media when $AgNO_3$ added to SGM only (Fig. 1 A, C, D and F). On the other hand, shoot regeneration frequency was significantly enhanced in proximal cotyledon and hypocotyl explants of both cultivars when $AgNO_3$ at the above concentrations was added to both SGM and RM (TE 3; Fig. 1 A, C, D, F). Distal hypocotyls explant was not responsive to any of the treatments tested in this study.

These observations suggest that the poor regeneration response found in control experiment (without $AgNO_3$, TE 1) of cucumber may be associated with ethylene production by the *in vitro* cultured cells or tissues. *In vitro* tissue cultures produce ethylene in sealed containers (Chi et al. 1991), which inhibits shoot regeneration (Chraibi et al. 1991) and impairs plant growth and development (Pua 1993). Curtis et al. (2004) also reported that the ethylene produced in *in vitro* radish culture might take part as a negative regulator to shoot regeneration. The slight improvement in shoot regeneration obtained from explants of TE 2 experiment compare to control (TE 1) indicates that the inhibition of ethylene production by $AgNO_3$ during seed germination and seedling growth could be stimulating more shoot primordia induction in both proximal cotyledon and hypocotyl explants of the SS and TG cultivars in comparison to the control. The significant enhancement of shoot regeneration in both cucumber cultivars obtained from experiment where $AgNO_3$ added to both

SGM and RM also supports hypothesis that the inhibition of ethylene production by $AgNO_3$, an ethylene inhibitor, in *in vitro* culture materials which eventually resulted higher shoot regeneration.

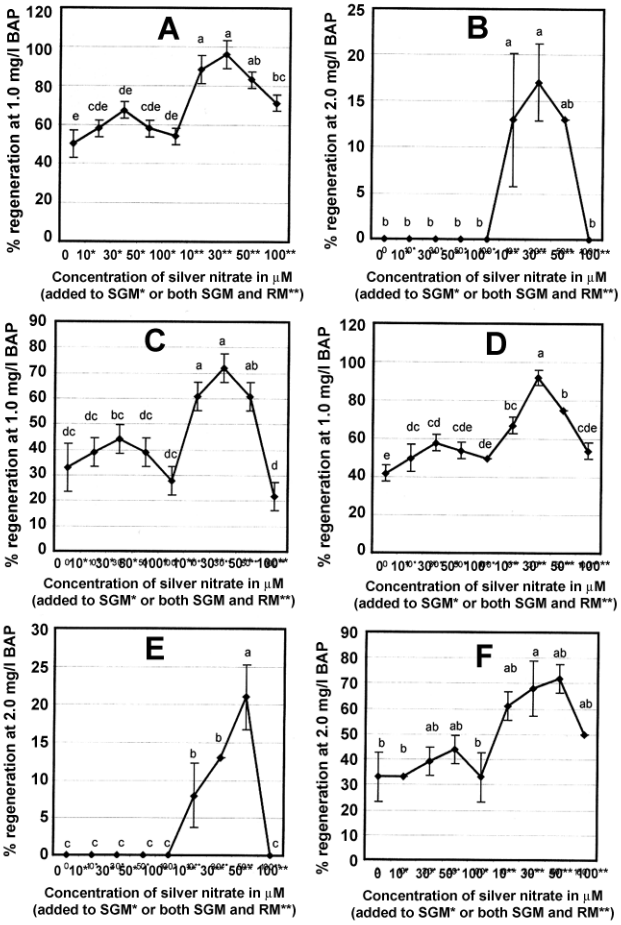

Fig. 1. Effect of $AgNO_3$ on shoot regeneration of proximal cotyledon (A/D), distal cotyledon (B/E) and proximal hypocotyls (C/F) of Spring Swallow/Tasty Green of cucumber. (different letters on the top of standard error bar differ significantly ($P < 0.05$).

Distal cotyledon of SS and TG did not produce any shoot primordium when $AgNO_3$ was not included to the both SGM and RM (TE 1). No shoot primordium was also observed from the same explants of SS and TG cultivars when $AgNO_3$ added to SGM (TE 2) only. On the other hand, the addition of 10, 30, and 50 µM of $AgNO_3$ to both the SGM and RM (TE 3) initiated shoot regeneration (Fig. 1, B and E). It suggests that addition of ethylene inhibitor, $AgNO_3$, to both media, SGM and RM, is a prerequisite to regenerate shoots from recalcitrant explants. The initiation of shoot regeneration from recalcitrant explants may be due to the

inhibitory activity of $AgNO_3$ on ethylene production and this action probably causes by an interference with ethylene binding during *in vitro* cell differentiation as suggested by other workers (Chraibi et al. 1991; Pua 1993). Distal hypocotyls of both cultivars, on the other hand, were unresponsive even though $AgNO_3$ added to both media (data not shown). Palmer (1992) reported similar response in hypocotyls explants of *Brassica campestris*.

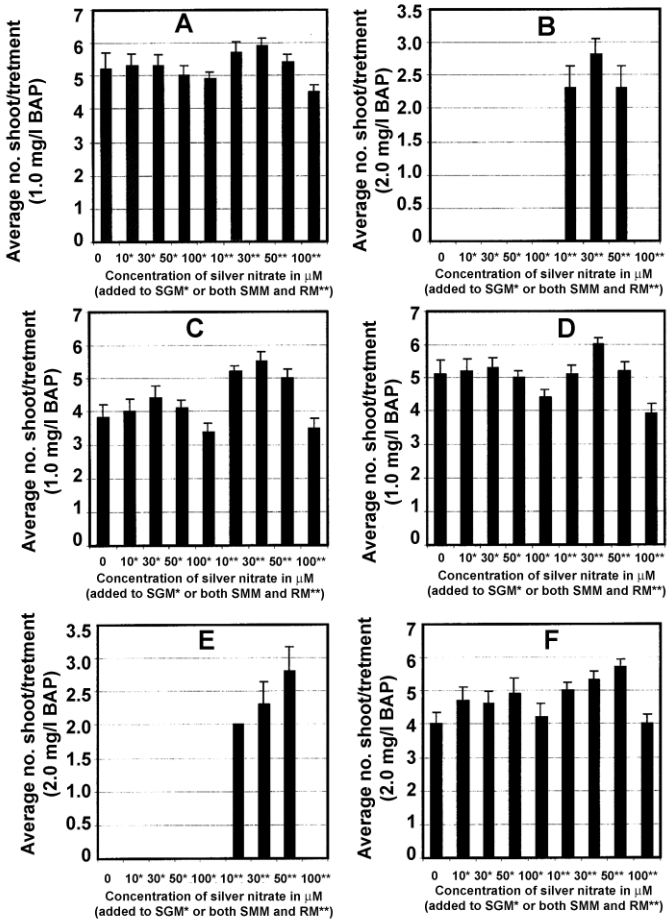

Fig. 2. Effect of $AgNO_3$ on number of shoots regenerated from proximal cotyledon (A/D), distal cotyledon (B/E) and proximal hypocotyls (C/F) of Spring Swallow/Tasty Green of cucumber.

Among the four $AgNO_3$ concentrations, 30 and 50 µM, added to both media, initiated significantly better response in relation to shoot regeneration and induced higher number of shoots per explant in both SS (Fig. 3a) and TG explants. $AgNO_3$ at 30 µM combined with 1.0 mg/l BAP was the best treatment for shoot regeneration i.e. 96 and 92% in proximal cotyledon of SS and TG, respectively (Fig. 1A and D). The same treatment was also found to be best for

proximal hypocotyl explants (72%) of SS (Fig. 1 C). On the other hand, $AgNO_3$ at 50 μM combined with 2.0 mg/l BAP proved to be the best treatment for shoot regeneration for proximal hypocotyl (72%) of TG (Fig. 1 F). The same treatment was also best for maximum shoot regeneration in distal cotyledon of TG (Fig. 1 E). However, the $AgNO_3$ concentration (30 μM) together with BAP at 2.0 mg/l induced maximum shoot regeneration (17%) in distal cotyledon of SS (Fig. 1 B). These results indicate that the shoot regeneration is explant and cultivar dependent. On average, two-fold or higher shoot regeneration was obtained from proximal cotyledon and hypocotyl explants of both cultivars when $AgNO_3$ was added to SGM as well as RM in comparison to the control, without $AgNO_3$ (Fig. 1A-F). Similar enhancement in shoot regeneration was observed in *Cucumis melo* (Roustan et al. 1992), buffalograss (Fei et al. 2000), cassava (Zhang et al. 2001) and *Brassica* species (Eapen and George 1997) by $AgNO_3$, added to regeneration medium only. Our previous study also showed that regeneration enhancement could be attained in cucumber when we added $AgNO_3$ to the regeneration medium (RM) only (Mohiuddin et al. 1997). However, our current study revealed that regeneration efficiency could be increased further in cucumber when we supplement $AgNO_3$ to both SGM and RM. It indicates that inhibitory effect of $AgNO_3$ in regeneration stage alone is not only crucial, however, in conjunction with seed germination stage too, its addition is mandatory. $AgNO_3$ level more than 50 μM was not useful.

The lower rate of shoot regeneration efficiency observed in hypocotyl explants compared to cotyledon (mentioned above) is in general agreement with published reports. For example, hypocotyls of *Brassica campestris* are less responsive than cotyledons (Chi and Pua 1989; Palmer 1992). Curtis et al. (2004) also observed such variation in cotyledon and hypocotyl explants of radish may imply different sensitivities in two explants towards a reduction in ethylene biosynthesis. Over 90% regeneration response of proximal cotyledons of both cultivars of cucumber makes the explant excellent target of genetic engineering.

The addition of $AgNO_3$ to both SGM and RM also showed an enhanced ability to produce higher number of shoots per explant in both cultivars (Fig. 2 A-F). The number shoot production ability was highly correlated with the regeneration response of each explant type × media combinations. The shoot production ability of proximal hypocotyls increased significantly when $AgNO_3$ was added to both SGM and RM compared to the control (Fig. 2 C and F). However, the effect was not pronounced for the proximal cotyledons (Fig. 2 A and D). It suggests that the addition of $AgNO_3$ to both media is also helpful for less responsive explants.

`We observed that the high concentration of $AgNO_3$ (100 μM) was inhibitory rather than stimulatory to shoot regeneration in distal cotyledons of both SS and

TG cultivars (Fig. 1, B and E). It suggests that this concentration has toxic effect on this explant. Similar results have also been observed in sunflower callus cultured in the presence of 40 and 80 μM of $AgNO_3$ (Chraibi et al. 1991). High concentration of $AgNO_3$ was also harmful in muskmelon shoot regeneration (Roustan et al. 1992).

Fig. 3. Multiple shoots regenerated from proximal cotyledon of SS in presence of $AgNO_3$ at 30 μM (A) and successfully acclimatized cucumber plant sets fruit (B).

Regenerated shoots were rooted in MS containing 0.5 mg/l IBA. Root primordia began to develop after six - eight days in culture. Rooted plantlets were successfully acclimatized to ambient humidity levels and subsequently transferred to soil in pots. The plants were normal and produced normal flowers and set fruits (Fig. 3b).

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