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Effect of Polyamines and Silver Nitrate on the High Frequency Regeneration from Cotyledon Explants of Bottle Gourd (*Lagenaria siceraria*; sp. asiatica)

Saha Shyamali and Kazumi Hattori
Laboratory of Plant Genetics and Breeding, Graduate School of Bioagricultural Sciences,
Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Abstract: In this study, we have investigated the effect of polyamines (PA) and silver nitrate ($AgNO_3$) on the high frequency regeneration from cotyledon explants of bottle gourd containing Murashige and Skoog (MS) media supplemented with different kind of Cytokinin alone or in the combination. Synergistic effect of kinetin (1 mg L^{-1}) and benzyl adenine (BA) (2 mg L^{-1}) itself showed highest shoot regeneration (80.6%) efficiency than BA or Kinetin alone in cotyledon explants of bottle gourd without adding $AgNO_3$ or PAs. We have also observed that PAs and $AgNO_3$ show their sensitivity on the regeneration, which is hormonal dependent. Regenerated shoots were rooted in half strength MS media containing 0.1 mg L^{-1} IAA.

Key words: *Lagenaria*, silver nitrate, polyamines, cytokinins, regeneration

INTRODUCTION

The genus *Lagenaria* belonging to the family of cucurbitaceae is an important vegetable in the tropics, subtropics and many other regions around the world. Morphological analysis and archeological evidence suggest the oceanic dispersals of wild bottle gourd fruit from Africa to Asia and America by 10000-15000 BP followed by independent domestication on all three Continents (Deena *et al*., 2001). The young fruits and tender tips of fast growing vines are eaten as a green vegetable. This vegetable contains high moisture carbohydrates, proteins, fats, fibers, minerals and vitamins, especially vitamin C and B complex (Heiser, 1979). With maturation, the rind becomes very hard and durable; the fruit is used to make utensils or fabricated into works of art (Yamaguchi, 1980). Bottle gourd have commercially important, especially in Japan, Korea, the Mediterranean area and Southern Europe, as they are being used as root stocks for grafting of watermelon and other cucurbits crops (Lee and Oda, 2003).

Cytokinins are known as ethylene inducing plant hormones and play an essential and crucial role in various aspects of plant growth and development of morphogenesis. In recent years there has been increasing evidence that the occurrence of morphogenesis in cultured plant cell may be associated with ethylene. Ethylene stimulated shoot morphogenesis in rice callus (Adkins *et al*., 1990) and *Pinus radiata* cotyledons (Kumar *et al*., 1987), embryogenesis from anther culture of Barley (Cho and Kasha, 1989) and flower bud formation in tobacco explants (Smulders *et al*., 1990). On the contrary, inhabitor ethylene action ($AgNO_3$) in *Nicotiana* (Huxter *et al*., 1981), *Triticum* (Purnhauser *et al*., 1987), *Zea mays* (Songstad *et al*., 1988) and *Brassica* (Chi *et al*., 1991). The inhibitory effects of ethylene can be prevented by inhibition of ethylene action and bio synthesis (Pua, 1993). However the role of ethylene in plant cells and tissues grown *in vitro* is not well understood. Cytokinins are also known to promote ethylene production several folds in many plants, at least partially through the increase in ACC synthase activities (Abeles *et al*., 1992). N6-benzyladenine (BA), a synthetic cytokinin, synergistically enhances ethylene production in the presence of IAA in Mungbean hypocotyls (Yoshii and Imaseki, 1982). Kinetin alone slightly stimulates ethylene production by etiolate seedling of several species, but a remarkable synergistic effect of Kinetin on IAA induced ethylene production has been observed (Fuches and Lieberman, 1968). It has not yet been proven that a plant hormone might operate a specific function by itself. On the contrary, there are several potential mutual iterating points between hormones (Coenen and Lomax, 1997), depending upon the plant species and tissue type (Scumulling *et al*., 1997). The possibility of different hormonal receptors controlling

Corresponding Author: Shyamali Saha, Laboratory of Plant Genetics and Breeding, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan
Tel: +81-052-789-4018 Fax: +81-052-789-4018

growth and development is another new question, as well as whether different hormone types may compete for a common receptor or at least operate in separate signaling pathways (Timpte *et al*., 1995). In addition, Polyamines (PA) have been implicated in somatic embryogenesis and organogenesis *in vitro* in several plant species (Pua, 1999). In view of the fact that PAs and ethylene compete for the same precursor, S-adenosylmethionine for their synthesis (Evans and Malmberg, 1989), it was thought that their possible interactions and/or mutual regulation might play an important role in plant morphogenesis *in vitro*.

To address these questions, in this study, we investigated the capacity of shoot regeneration in different cytokinins in response to ethylene inhibitor ($AgNO_3$) and PAs from cotyledon explants of bottle gourd.

MATERIALS AND METHODS

Explants preparation and phytohormones: Decoated seeds of Barsa-F were sterilized by soaking in 70% ethanol for 1 min followed by 45 min in 20% Sodium hypochlorite (1% a.i) containing 0.2% Tween-20, rinsed 4 times with sterile distilled water and finally soaking in autoclave sterile water for 2 h. Sterilized seeds were placed on germination medium containing MS basal salts and vitamins, 2% (w/v) sucrose. The pH of the medium was adjusted to 5.8 before the addition of the 0.8% agar (INA AGAR BA-30). Cotyledon explants of seedling at various ages, ranging from 2 to 14 days were tested for their regeneration capacity on MS medium containing 2 mg L^{-1} BA and 1 mg L^{-1} kinetin combination. Four days proximal part of cotyledon were isolated from seedlings and cultured on MS basal medium supplemented with different level of BA and kinetin alone or both combinations to optimize the regeneration %, bud proliferation, number of shoot per explants and shoot elongation. Seed germination and all the cultures including shoot initiation and shoot growth cultures were maintained 27±1°C with 16 h photoperiod.

Silver nitrate and exogenous polyamines: The effect of ethylene action inhibitor, $AgNO_3$ of different concentrations viz. 5, 10 and 15 μM on BA or kinetin alone or their synergistic combination was examined on the regeneration potential of bottle gourd. The role of polyamines was also investigated by culturing explants on BA and kinetin alone or their synergistic combination supplemented with putrescine 5, 10, 15, 20 mM, respectively or spermidine 1, 2, 3, 4 μM, respectively on regeneration. Silver nitrate, putrescine and spermidine were sterilized by filtration and added to the medium after autoclaving.

Experimental design and data analysis: Each treatment was consisted of 4 explants with 8 replicates and each experiment was repeated three times. Data on the regeneration percentage, number of shoots per explants and shoot length were statistically tested by analysis of variance (ANOVA). The differences among the means were analyzed by Duncan's Multiple Range Test (DMRT) at 5% level of significance.

Root induction: Elongated young shoot were isolated from the cotyledon explants and cultured on a half strength MS medium containing 0.1 mg L^{-1} IAA for root induction.

RESULTS

Explants age: Age of plantlets greatly influenced the regeneration of Cotyledon explants of bottle gourd is shown in Fig. 1 and results are shown in Table 1. Cotyledon explants of various ages were tested for their organogenic potential for multiple shoot induction as well as regeneration. Shoot differentiation frequency varied remarkably when cotyledon explants from seedling of different ages were cultured on the MS medium supplemented with 2 mg L^{-1} BA and 1 mg L^{-1} kinetin. Proximal part of explants from 4 days seedling, gave highest (80.6%) regeneration compared to that obtained from 2, 7, 10 and 14 days (Table 1). Proximal part of cotyledon showed higher regeneration than distal part for all the ages tested.

Effect of cytokinins on regeneration: Different kinds of cytokinin and concentration were tested for their organogenic potential on regeneration from four days

Fig. 1: The cotyledon explants of different ages of bottle gourd seedling of genotype Barsa-F; 2, 4, 7, 10 and 14 days after sowing of the seeds *in vitro*

Table 1: Effect of explants age and explants types (proximal part % or distal part %) on adventitious shoot regeneration

Explants age (days)	Proximal part (%)	Distal part (%)
2	31.7c	3.3e
4	80.6a	11.7d
7	46.6b	6.7ed
10	13.3d	1.67f
14	1.44f	1.1f

Values in column followed by different letters differ significantly at the 5% levels (Duncan's multiple range test)

Table 2: Effect of different plant growth regulators on shoot initiation, shoot elongation and shoot regeneration from cotyledon explants

BA (mg L^{-1})	Kinetin (mg L^{-1})	Regeneration (%)	Shoot/explants	Shoot length (cm)
1	0	9.33h	2.24±0.10cd	2.05±0.46d
2	0	25.33d	3.80±0.56ab	2.85±0.38c
3	0	20.00edfg	3.07±0.46b	2.34±0.41d
0	1	18.67edfgh	2.66±0.49bc	3.70±0.37a
0	2	24.00ed	2.80±0.56bc	3.36±0.28b
0	3	21.3edf	2.01±0.63d	3.45±0.39ab
2	1	80.60a	4.06±0.70a	1.18±0.34e
2	2	33.33c	1.01±0.20e	0.70±0.21f
2	3	49.33b	1.00±0.10e	0.50±0.25f

Values in column followed by different letters differ significantly at the 5% levels (Duncan's multiple range test)

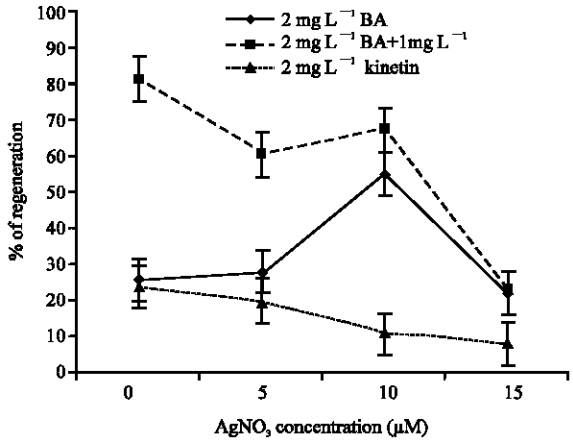

Fig. 2: Effect of different concentrations of $AgNO_3$ on shoot regeneration from proximal part of cotyledon explants of bottle gourd genotype as compared with BA, Kinetin alone or both. Bar indicates the standard error

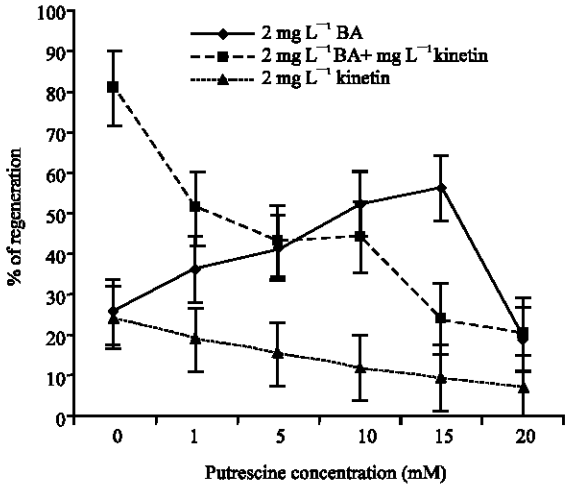

Fig. 3a: Effect of different concentrations of putrescine on shoot regeneration from proximal part of cotyledon explants of bottle gourd genotype as compared with BA, Kinetin alone or both. Bar indicates the standard error

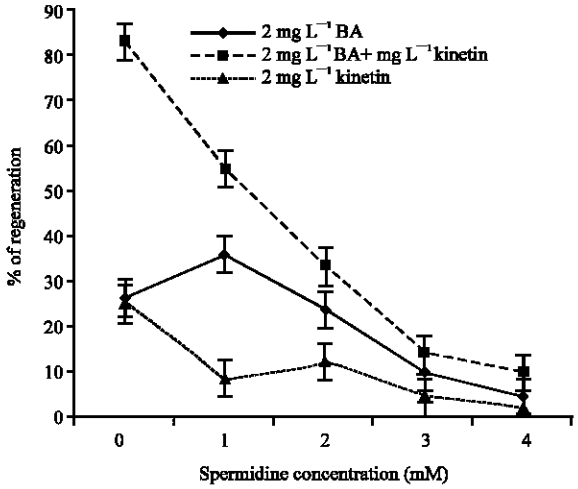

Fig. 3b: Effect of different concentrations of spermidine on shoot regeneration from proximal part of cotyledon explants of bottle gourd genotype as compared with BA, Kinetin alone or both. Bar indicates the standard error

proximal part of cotyledon explants of bottle gourd (Table 2). Highest number of regeneration (80.6%) and shoot/explants (4.06) was produced in MS medium supplemented with 2 mg L^{-1} BA and 1 mg L^{-1} kinetin combination (Fig. 4) among all the combination tested. Among the different concentrations of BA or Kinetin, 2 mg L^{-1} BA (25.33%) or 2 mg L^{-1} kinetin (24%) performed higher regeneration, respectively. The combination of 2 mg L^{-1} BA with higher concentrations of kinetin (2 and 3 mg L^{-1}) studied, bud proliferation state performed good but restricted to initiate the shoot, resulted in poor effect in terms of regeneration. In kinetin containing media, a positive effect on shoot elongation was found. Two milligram per liter kinetin proved to be best (3.7 cm) among all of the treatments (Table 2 and Fig. 4c).

Effect of $AgNO_3$ and polyamines on regeneration: The effect of $AgNO_3$ (Fig. 2) and Polyamines (Fig. 3a and b) was tested for their organogenic potential on regeneration in MS media containing BA and kinetin alone or both. There were no significant effect found on regeneration

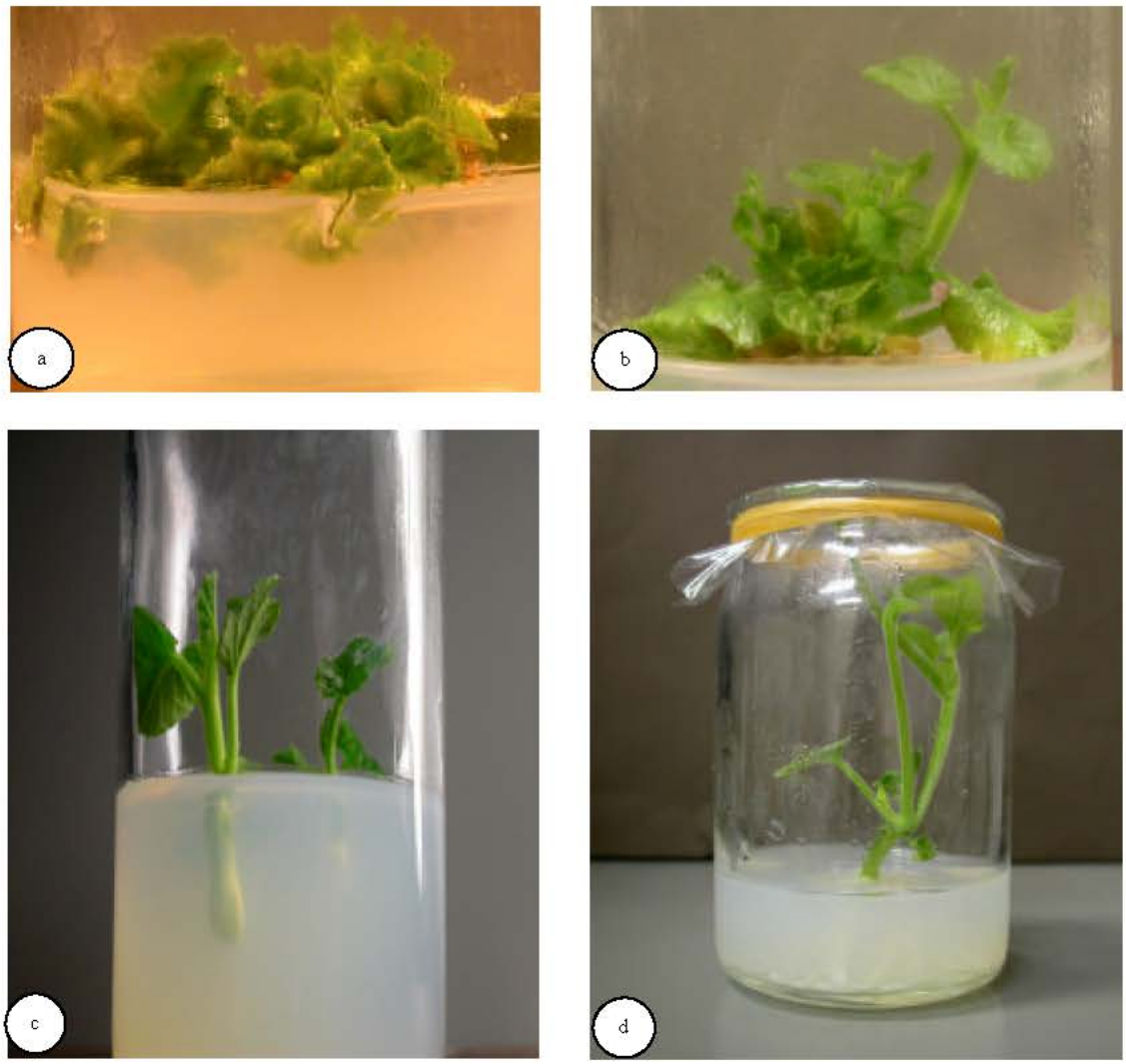

Fig. 4: Regeneration of shoots from the cotyledon explants of bottlegourd. (a) bud proliferation within 2 weeks of culture at 2 mg L^{-1} BA and 1 mg L^{-1} kinetin medium, (b) Shoot initiation at 3 weeks of culture in MS medium containing 2 mg L^{-1} BA an kinetin combination, (c) shoot elongation in 2 mg L^{-1} kinetin media and (d) root induction

improvement in MS media containing 2 mg L^{-1} kinetin or synergistic combination of 2 mg L^{-1} BA and 1 mg L^{-1} Kinetin in the presence of $AgNO_3$ (Fig. 2). But 2 mg L^{-1} BA (25.33%) was significantly increased the regeneration (54.67%) in the presence of $AgNO_3$ (10 μM). The application of PAs to the regeneration medium did not produce any significant improvement of regeneration on 2 mg L^{-1} BA with 1 mg L^{-1} kinetin combination. The application of putrescine and spermidine at several concentrations was tested on the regeneration potential of cotyledon explants of bottle gourd on kinetin, BA alone or their synergistic combination. Putrescine and spermidine containing 2 mg L^{-1} kinetin or synergistic combination (2 mg L^{-1} BA and 1 mg L^{-1} kinetin) had no effect on regeneration improvement (Fig. 3a). BA containing media (25.33%) increased their regeneration in the presence of 1, 5, 10 and 15 mM putrescine i.e., 36, 41.33, 52 and 56%, respectively (Fig. 3a). One micro molar spermidine also increased the regeneration (34.67%) in the presence of BA (Fig. 3b). Putrescine and spermidine are more effective on BA but not in 2 mg L^{-1} kinetin or combination of 2 mg L^{-1} BA and 1 mg L^{-1} kinetin for regeneration. $AgNO_3$, Putrescine and spermidine showed a cytokinin dependent sensitivity on regeneration.

DISCUSSION

Cotyledon explants of various ages were tested for their organogenic potential for multiple shoot induction as well as regeneration. The highest shoot regeneration was found in the 4 days old seedling explants compared with others. Proximal part of cotyledon showed a higher frequency of regeneration when compared with the distal part (Fig. 1). Although plant growth regulator modifications during the culture period are important for the successful regeneration, the most determining factor for regeneration competence of bottle gourd was their optimal explants age. These results agree well with the reports of bottle gourd (Han *et al*., 2004), cucumber (Mohiuddin *et al*., 1997) and squash (Lee *et al*., 2003). A possible explanation is that the 4 days proximal part of young cotyledons are very active in physiology and are easily affected by environmental factors, such as, exogenous hormones.

According to the previous study of Han *et al*. (2004), only BA may be considered a crucial factor for adventitious shoot regeneration of bottle gourd and $AgNO_3$, a potent inhibitor improves the regeneration. The results obtained from our study are not only compatible with this finding, but also clearly indicates the use of $AgNO_3$ is more potent on BA. Concurrently it has no significant effect on kinetin or 2 mg L^{-1} BA and 1 mg L^{-1} kinetin combination for higher regeneration (Fig. 2). Similar results obtained from nodal explants of Pistachio (Ozden-Tokatli *et al*., 2005). They suggested that the medium containing BA could possibly release higher amount of ethylene than kinetin containing media. As a result, the inhibitory effect of $AgNO_3$ on ethylene action could be more significant on BA containing media for higher regeneration. So, our results strongly support it. But it is not clear yet, how the kinetin keeps a role on ethylene inhibition in the presence of BA *in vitro*. Shoot regeneration capacity of explants and stimulation of ethylene biosynthesis may vary; depending on the growth regulator used (Kumar *et al*., 1998).

The effect of polyamines on the regeneration was effective on BA medium. But kinetin and synergistic combination did not found any significant improvement on regeneration (2 mg L^{-1} BA and 1 mg L^{-1} kinetin) with polyamines (Fig. 3a and b). PAs play an important role in shoot regeneration from Passiflora leaves (Desai and Metha, 1985). However, in morphogenesis of different species or different explants, spermidine and putrescine have been observed to exhibit dissimilar effects (Zhu and Chen, 2004). Exogenously added PAs recover browning tissues into normal callus cultured by decreasing oxidative damage and improving plant regeneration by acting as plant growth substance in Pine as reported by Tang *et al*. (2004). The accumulative evidences of culture *in vitro* indicate that polyamines showed a cytokinin dependent sensitivity on regeneration. May be it is related with ethylene. The role of PAs has been reported in a wide range of biological and physiological processes, but the precise mode of PAs action is still unclear.

From the foregoing discussion it can be concluded that synergistic effect of 2 mg L^{-1} BA and 1 mg L^{-1} kinetin itself performed best on the regeneration from cotyledon explants of bottle gourd. We report such an opposite effect of $AgNO_3$ and PAs within the same explants, which may be explained in terms of regeneration pattern induced by different exogenous growth regulators, likely resulting in changed sensitivity to the hormone dependent manner. Similar results were found in Japanese variety, Yu-Gao but regeneration % found very poor compared to Barsa-F. Relatively little is known about the molecular mechanisms of cross-talk and integration between hormone responses on regeneration. A few downstream genes are known to modulate or integrate different hormonal signals, deserve further investigations.

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