The interaction between plant hormones throughout plant regeneration and development is complex. In the present study, we extended our investigation and disclosed the capacity of de novo shoot organogenesis from cotyledon explants of bottle gourd in relation to plant hormones and ethylene inhibitor. Synergistic effect of kinetin and benzyl adenine (BA) enhanced the shoot regeneration efficiency (80.6%) from cotyledon explants of bottle gourd, compared with BA or kinetin used separately, even in the presence of AgNO₃. The use of BA or kinetin separately or in synergistic combination led to a characteristic brownish color of the explants at the bud formation stage which was related to ethylene production. In the cotyledon explants cultured on MS medium supplemented with kinetin, the production of ethylene was lower than when BA or the combination of BA and kinetin was used. However, the regeneration capacity was very low and the emergence of shoot buds was delayed. BA promoted ethylene production while the synergistic effect of kinetin and BA decreased ethylene production and played a role in high frequency of regeneration from cotyledon explants of bottle gourd. The physiological relevance of the hormonal interaction in relation to the regulation of ethylene action and effect on regeneration was discussed.

Key Words: Lagenaria, ethylene, cytokinin, regeneration.

Introduction

The genus Lagenaria belonging to the family Cucurbitaceae is important in the tropics, subtropics and many other regions around the world. Bottle gourd is an edible, medicinal and utilitarian domesticated cucurbit with an ancient old tropical distribution (Deena et al. 2001). The young fruits and tender tips of fast growing vines are eaten as a green vegetable. With maturation, the rind becomes very hard and durable; the fruit is used to make utensils or artifacts (Yamaguchi 1980). Bottle gourd has a commercial importance, especially in Japan, Korea, the Mediterranean area and Southern Europe, as it is being used as a rootstock for the grafting of watermelon and other cucurbit crops, providing increased vigor to young plants vigor (Lee and Oda 2003). Compared with cucumber (Cucumis sativus), melon (Cucumis melo) and watermelon (Citrullus vulgaris), less attention has been given to the improvement of Lagenaria.

Cytokinins, known as ethylene-inducing plant hormones, play an essential role in various aspects of plant growth and development. Plants that are wounded or exposed to environmental stresses or pathogen attacks usually exhibit an enhanced ethylene production.

In recent years, it has become increasingly evident that morphogenesis in cultured plant cells may be associated with ethylene production. Ethylene stimulated shoot regeneration 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 biosynthesis (Pua 1993). However the role of ethylene in plant cells and tissues grown in vitro has not been fully elucidated. Cytokinins are also known to increase ethylene production several fold in many plants, at least partially through the increase in ACC synthase activity (Abeles et al. 1992). N6-benzyladenine (BA), a synthetic cytokinin, synergistically enhanced ethylene production in the presence of IAA in mungbean hypocotyls (Yoshii and Imaseki 1982). Kinetin alone slightly stimulated ethylene production by etiolated...
seedlings in several species (pea, mungbean) while the remarkable synergistic effect of kinetin on IAA-induced ethylene production has been observed (Fuches and Lieberman 1968). It has not yet been confirmed that a plant hormone might fulfill a specific function by itself. On the contrary, there are several potential mutual interactions between hormones (Coenen and Lomax 1997), depending the plant species and tissue type (Schmulling et al. 1997). The possibility that different hormonal receptors control growth and development has been considered and it remains to be determined whether different hormone types may compete for a common receptor or at least operate in separate signaling pathways (Timppe et al. 1995).

To address the above problems, the present study was carried out and revealed the capacity of de novo shoot organogenesis from cotyledon explants in bottle gourd in vitro in relation to the effect of ethylene, ethylene inhibitor(s) and plant hormones. In the present communication, we reported for the first time, the stimulatory effect of BA and kinetin on adventitious shoot organogenesis, leading to the high frequency of plant regeneration from cotyledon explants of bottle gourd in relation to ethylene production.

Materials and Methods

Explant preparation and plant hormones

Decoated seeds of Barsa-F supplied by East West seed company (Bangladesh) were sterilized by soaking in 70% ethanol for 1 minute followed by 45 minutes in 1% sodium hypochlorite containing 0.2% Tween-20. They were rinsed 4 times with sterile distilled water and finally soaked in autoclaved sterile water for 2 hours. Sterilized seeds were placed on a germination medium containing MS basal salts, vitamins and 2% (w/v) sucrose. The pH of the medium was adjusted to 5.8 before the addition of 0.8% agar. Cotyledon explants of seedlings at various ages ranging from 2 to 10 day-old were tested for their regeneration capacity on MS medium containing a combination of 2 mg/l BA and 1 mg/l kinetin. Proximal part of the cotyledon was isolated from the seedlings (4-day-old) and cultured on MS basal medium supplemented with different levels of BA or kinetin or in combination to optimize the regeneration percentage, bud proliferation, number of shoots per explant and shoot elongation. Seed germination and all the cultures including bud proliferation, shoot initiation and shoot growth were maintained at 27±1°C under a 16-hour photoperiod.

Ethylene measurement

Explants excised from bottle gourd cotyledons were put into 50 ml Erlenmeyer flasks and were grown in different culture media. Each flask was sealed with an airtight rubber stopper and incubated for 30 min. Ethylene measurement was performed at different intervals (0, 2, 5 and 7 days). Each treatment consisted of 8 explants with 3 replications. One milliliter of sample in the head space of an Erlenmeyer flask was removed with a syringe and injected into a GL Science model GC-353 gas chromatograph, equipped with a fused silica capillary column (Neutra bond-1). Ethylene was quantified by comparison with an ethylene standard.

Effect of silver nitrate (AgNO₃) on explant regeneration

The effect of the ethylene action inhibitor, AgNO₃ on BA or kinetin used separately or in synergistic combination was examined in the regeneration of bottle gourd. The role of AgNO₃ was investigated by culturing explants on MS media supplemented with BA or kinetin separately or in synergistic combination, using AgNO₃ at 0, 5, 10 and 15 µM concentrations, respectively. AgNO₃ was sterilized by filtration and then added to the medium after autoclaving.

Data analysis

Each treatment consisted of 4 explants with 8 replications and each experiment was repeated two times. Data on the bud proliferation state, percentage of regeneration, number of shoots per explant and shoot length were statistically analyzed using the GLM procedure of SAS package version 8.2 and Tukey’s studentized range test at 5% level of significance. Shoot regeneration efficiency was calculated as the percentage of the number of explants that regenerated shoots out of the total number of explants cultured in each treatment. Bud proliferation stage was observed in explants after two weeks of culture in each treatment prior to shoot initiation and estimated visually, based on the swelling of the proximal end from where the number of shoots (high ++, moderate ++ and low +) was initiated in each treatment. Shoot number per explant was calculated as the number of regenerated shoots from each explant with bud in each treatment.

Root induction

Elongated young shoots were isolated from the cotyledon explants and then cultured on half-strength MS medium containing 0.1 mg/l IAA for root induction.

Results

Optimal conditions of explant age and type for culture

Age of the explants markedly influenced the regeneration of the cotyledon explants of bottle gourd (Fig. 1). Shoot differentiation frequency varied remarkably when cotyledon explants from seedlings at different ages were cultured on MS medium supplemented with 2 mg/l BA and 1 mg/l kinetin. The use of the proximal part of the explants from 4-day-old seedlings resulted in the highest (80.6%) regeneration percentage compared with that of the explants obtained from 2, 7 and 10-day-old seedlings. The use of the proximal part of the cotyledon led to a higher regeneration percentage than that of the distal part for all the ages tested. Proximal part of the cotyledon explants of 4-day-old seedlings was found to be the optimal source for culture.
Synergistic effect of kinetin and BA on regeneration in bottle gourd

Effect of cytokinins on explant regeneration

Different kinds of cytokinins and concentrations were tested for their organogenic potential in the regeneration from the proximal part of cotyledon explants of 4-day-old of bottle gourd (Table 1). Higher bud proliferation was observed on MS medium containing a combination of 2 mg/l BA and 1 mg/l kinetin and were evaluated after 5 weeks of culture. Values in bars followed by different letters differ significantly by Tukey’s studentized range test at 5% level.

Characteristic brownish coloration of explants and ethylene production at bud formation stage

The use of BA or kinetin separately or in synergistic combination in MS media led to a characteristic brownish coloration of the explants at the bud formation stage after 7 days of culture (before subsequent subculture) (Fig. 2). Four-day-old proximal explants showed a more pronounced brownish coloration on MS media supplemented with 2 mg/l BA at the bud proliferation stage (Fig. 2a) after 7 days of culture. No brownish phenotype was observed on media containing kinetin, while shoot bud emergence was delayed (Fig. 2c). A characteristic moderate brownish coloration (Fig. 2b) was observed in the synergistic combination (2 mg/l BA and 1 mg/l kinetin). Ethylene measurement was tested at different age of explant (0-, 2-, 5- and 7-day-old) when MS medium containing BA or kinetin separately or in combination was used (Fig. 3). After 7 days, the use of containing BA resulted in a higher ethylene production (5.5 ng/l/hr) from cotyledon explants, compared with the synergistic combination of 2 mg/lBA and 1 mg/l kinetin (2.9 ng/l/hr). A very low level of ethylene was produced when kinetin was used throughout the study. Initially (2 days), the synergistic combination led to a higher ethylene production from the cotyledon explants of bottle gourd compared with the use of BA, but the production gradually decreased after 5 to 7 days of culture. A positive relationship was found between ethylene production and tissue browning in the cotyledon explants of bottle gourd at the bud proliferation stage (Fig. 2 and Fig. 3).

Table 1. Effects of plant hormones on bud formation, shoot initiation, shoot elongation and shoot regeneration from cotyledon explants of bottle gourd

<table>
<thead>
<tr>
<th>BA (mg/l)</th>
<th>Kinetin (mg/l)</th>
<th>Bud proliferation(1)</th>
<th>Percentage of explants with shoots</th>
<th>No. of shoots per explant</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>++</td>
<td>9.3 d</td>
<td>2.2±0.1 cd</td>
<td>2.1±0.5 c</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>++</td>
<td>25.3 cd</td>
<td>3.8±0.6 a</td>
<td>2.9±0.4 b</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>++</td>
<td>20.0 cd</td>
<td>3.1±0.5 b</td>
<td>2.3±0.4 c</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>+</td>
<td>18.7 cd</td>
<td>2.7±0.5 bc</td>
<td>3.7±0.4 a</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>+</td>
<td>24.0 cd</td>
<td>2.8±0.6 bc</td>
<td>3.4±0.3 a</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>++</td>
<td>21.3 cd</td>
<td>2.0±0.6 d</td>
<td>3.5±0.4 a</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>+++</td>
<td>80.6 a</td>
<td>4.1±0.7 a</td>
<td>1.2±0.4 d</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>+++</td>
<td>33.3 bc</td>
<td>1.0±0.2 e</td>
<td>1.0±0.2 e</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>+++</td>
<td>49.3 b</td>
<td>1.0±0.1 e</td>
<td>1.0±0.3 e</td>
</tr>
</tbody>
</table>

(1) Bud proliferation was scored after two weeks of culture according to the level of proliferation (+++ high, ++ moderate and + low).

Values in a column followed by different letters differ significantly by Tukey’s studentized range test at 5% level of significance.
Effect of AgNO₃ on different kinds of cytokinins

The effect of AgNO₃ (Fig. 4) was tested for its organogenic potential for regeneration on MS media containing BA or kinetin separately or in combination. No significant beneficial effect was found on regeneration enhancement on MS medium containing 2 mg/l kinetin or a synergistic combination of 2 mg/l BA and 1 mg/l kinetin in the presence of AgNO₃, compared with the control (Fig. 4). However, the use of 2 mg/l BA (25.3%) significantly increased the regeneration percentage (54.7%) in the presence of AgNO₃ (10 μM). These results indicated that AgNO₃ showed a cytokinin-dependent sensitivity in regeneration.

Discussion

In the bottle gourd, cotyledon explants from seedlings at various ages were tested for their organogenic potential for multiple shoot induction as well as regeneration. The highest level of shoot regeneration occurred in the 4-day-old seedling explants compared with others. Proximal part of the cotyledon induced a higher frequency of regeneration, compared with the distal part (Fig. 1). Although plant growth regulator modifications during the culture period are important for successful regeneration, the major determining factor for regeneration competence was associated with optimal explant age. These results were in good agreement with previous reports on bottle gourd (Han et al. 2004), cucumber (Mohiuddin et al. 1997) and squash (Lee et al. 2003). A possible explanation is that the proximal part of young cotyledons of 4-day-old seedlings was very active physiologically and was easily affected by environmental factors, such as the presence of exogenous hormones.

Bud proliferation, number of shoots per explant, shoot regeneration and shoot elongation varied depending on the kind and concentration of the cytokinins used (Table 1). The use of a combination of cytokinins (BA and kinetin) enhanced higher bud proliferation, compared with the use of cytokinins separately. Kinetin was less effective than BA in terms of bud proliferation as well as shoot bud induction, but exerted a beneficial effect on shoot elongation (> 3.36 cm). Promotion of shoot bud differentiation in combined use of

![Figure 2](image1.jpg)

Fig. 2. Effect of use of either cytokinin separately or in synergistic combination on characteristic coloration of the explant after 7 days culture at the bud formation stage. (a) Buds showed a pronounced brownish coloration on MS medium containing 2 mg/l BA. (b) Buds showed a moderate brownish coloration on MS medium containing a combination of 2 mg/l BA with 1 mg/l kinetin. (c) No brownish coloration appeared when 2 mg/l kinetin was used.

![Figure 3](image2.jpg)

Fig. 3. Effects of plant hormones on ethylene production at different explant ages. Each treatment consisted of three replications with eight explants each. Vertical bars indicate standard error of means.

![Figure 4](image3.jpg)

Fig. 4. Effects of AgNO₃ on shoot regeneration from the proximal part of cotyledon explants of bottle gourd, compared with the use of BA or kinetin separately or in combination. Values in bars followed by different letters differ significantly by Tukey’s studentized range test at 5% level of significance.
BA and kinetin has been reported in *Feronia limonia* L. hypocotyl explants (Vyas et al. 2005) and cotyledonal explants (Hossain et al. 1994) as well as *Vigna radiata* L. (Gulati and Jaiwal 1990). The use of either cytokinin separately or in synergistic combination led to a characteristic phenotype in vitro after 7 days of culture at the bud proliferation stage before subsequent subculture (Fig. 2). BA led to a more brownish phenotypic coloration (Fig. 2a) than the combination of BA and kinetin (Fig. 2b). Browning of tissue was not observed when cotyledon explants were cultured on a medium containing kinetin (Fig. 2c). Similar results were reported in *Pistacia vera* cv. Kirmiz, where browning of the explants was commonly observed on a medium containing BA as cytokinin. In contrast, the extent of browning was negligible on a medium containing BA and kinetin (Ozden-Tokatli et al. 2005). Ethylene enhanced the activities of peroxidases and bound polyphenol oxidase, associated with the metabolism of phenolic products, and tissue browning was reported in *Hevea brasiliensis* (Housti et al. 1992). Our results are also consistent with these findings on ethylene production (Fig. 3) as cotyledon explants on media containing BA promoted ethylene production. However, kinetin led to a lesser ethylene production from cotyledon explants of bottle gourd after 7 days of culture in vitro. Our results revealed that the combined effect of kinetin and BA in the growth medium induced a lower ethylene production than that of BA only, a long with a decreased brownish coloration, suggesting the existence of cell differentiation (Fig. 2 and Fig. 3). Therefore, the interaction between kinetin (1 mg/l) and BA (2 mg/l) contributed significantly to shoot regeneration (80.6%) among all the combinations studied (Table 1). The combination of 2 mg/l BA with higher concentrations of kinetin (2 mg/l, 3 mg/l) led to adequate bud proliferation but to restricted initiation of shoots from buds, hence the limited effect in terms of regeneration (Table 1). On the media containing kinetin, a beneficial effect on shoot elongation was observed on the use of 2 mg/l kinetin was found to be optimal for shoot elongation (3.7 cm), among all the treatments (Fig. 2c). Ethylene also acts as an endogenous regulator of various morphogenic processes, including the determination of the size of organs (Bleecker et al. 1998), and inhibition is often associated with the action of ethylene and/or peroxidases (Abeles et al. 1992). Our results are consistent with this observation, since shoot length significantly increased on media containing kinetin.

According to the previous study of Han et al. (2004), only BA was considered to be an essential factor for adventitious shoot regeneration of bottle gourd and the use of AgNO₃, a potent ethylene action inhibitor, increased the regeneration percentage from 37.5% to 52.4%. The results obtained in our study are compatible with this finding, and also clearly indicate that the use of AgNO₃ is more effective on BA. Concurrently, AgNO₃ does not exert a significant effect on the use of kinetin or a combination of 2 mg/l BA and 1 mg/l kinetin to achieve a higher regeneration percentage (Fig. 4). Similar results were obtained from nodal explants of pistachio by Ozden-Tokatli et al. (2005), who suggested that a medium containing BA could possibly release a higher amount of ethylene than a medium containing kinetin. As a result, the inhibitory effect of AgNO₃ on ethylene action could be more significant on media containing BA to achieve a higher percentage of regeneration. It remains to be determined how kinetin plays a role in 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).

Based on the foregoing discussion, it can be concluded that BA is necessary for shoot bud formation and kinetin plays a vital role in regeneration in relation to ethylene production. Therefore the synergistic effect of BA and kinetin was optimal on the regeneration from cotyledon explants of bottle gourd. The differences in the effects of AgNO₃ application within the same explants, may be explained in terms of regeneration pattern induced by different exogenous growth regulators, likely resulting in changes in to the hormone-dependent sensitivity in relation to ethylene production. A similar phenomenon was reported in the Japanese variety, Yu-Gao (Peacock seed company) although the regeneration percentage was very low, compared with that of Barsa-F. Relatively little information is available about the molecular mechanisms of cross-talk and integration between hormone responses in relation to regeneration. The present study may thus open a new avenue for the improvement of shoot regeneration, based on the role of ethylene associated with hormonal interaction in bottle gourd.
Acknowledgements

The authors are grateful to East West Seed (Bangladesh) Ltd. for the supply of seeds. The authors thank Hori Information Science of Promotion Foundation for providing a research grant for this work. Special thanks are due to Makio Kato, Plant Genetics and Breeding Lab., Nagoya University, for computer support.

Literature Cited


