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Effect of Growth Regulators on Plantlet Regeneration and Bulbing in Onion (*Mum cepa* L.) *in vitro*

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Abstract: The experiment was conducted to study the effect of growth regulators on plantlet regeneration and bulbing of onion in vitro. Results from the experiment showed that both plantlet regeneration and bulbing were greatly affected by growth regulators or their combinations. It was found that shoot induced from twin scale in 0.1 mg/l NAA was with a percentage of 93% and per explant shoot rate of 0.84. A combination of 0.1 mg/l NAA + 2 mg/l $4PU_{30}$ induced 89% shoot regeneration from twin scale. The highest per explant shoot (1.25), however, was observed in 0.1 mg/l NAA + 1 mg/l $4PU_{30}$. There was no significant difference between the effect of combining NAA + BA and NAA + $4PU_{30}$ on shoot production. The highest root percentage (89%) in twin scale was obtained with a combination of 0.1 NAA + 3 mg/l BA, and highest per explant root (1.06) was observed in 0.1 mg/l NAA + 1 mg/1 BA treatment. The effect of $4PU_{30}$ and sucrose on inducing bulb formation independently was also studied. The highest bulb percent (84%) was induced in 0.8 mg/l $4PU_{30}$. The mean bulb circumference at the same concentration was 2.6 cm after six weeks of culture. In sucrose treatment, the highest bulb percentage (81%) was observed in 100 g/l sucrose. Apparently, a slightly higher (2.9 cm) mean bulb circumference was observed compared to $4PU_{30}$. There was, however, no significant difference in bulb circumference between the two treatments.

Key words: Onion (*Allium cepa*), tissue culture, bulbing in vitro, growth regulator

Introduction

An interesting trait of most edible Allium species is their property to form a bulb as storage, preservation and spreading means. Onion (*Affium cepa* L.) belongs to a family of which several tissues express a high regeneration potential, (Hussey, 1976), therefore *in vitro* technique has been introduced to it for vegetative multiplication (Hussey and Falavigna, 1980). Meanwhile, healthy maintenance and rapid multiplication of male-sterile and other breeding lines by vegetative propagation would be highly advantageous for onion breeding, and which has recently been shown that such propagation may be carried out by serial production of *in vitro* shoots. Recently, studies on onion crop have also been carried out in many aspects such as physiology of growth and development, resistance to pest and fungi. The potential advantage of using tissue culture for more rapid propagation of onion with built-in disease protection as led to work aimed at developing a suitable *in vitro* method (Hussey, 1975). Whereas, the greatest challenge for the investigators is to understand the mechanisms of the factors affecting bulb formation.

Bulbing in onion has been studied for *years* by agronomist and plant physiologists in onion (Guo, 1996). Bulbing *in vitro* was encountered occasionally as an uncontrolled and limiting factor to micropropagation. Bulb formation with micropropagatd plants also allows conversation possibilities in onion growing area (Kahane *et al.,* 1997). Therefore, *in vitro* technique appears very useful to study this complex process of bulbing in a model way. Although there has been pervious work on onion *in vitro* with regard to callus production and shoot regeneration from twin scales, no method has been developed to enable plant mass propagation. In fact, no controlled bulbing has been obtained from tissue cultures in onion so far. However, there is a real need of clones in economically important Allium crops like garlic and onion for breding program. A successful multiplication process for several authors from basal parts of one bulb have described a single cycle of regeneration cultivated *in vitro* on MS medium supplemented with NAA and BA or kintein. But no report has been found to test the effect of $4PU_{30}$, a chemical with very high activity of cytokinin. Even so onion tissues were capable of shoot regeneration for only a limited length of time. Bulbiet formation, plantlet dormancy, and a decrease in regenerative ability or in multiplication rate have been previously reported as limiting factors to micropropagation of onion. Our aim was to overcome these problems to produce rapidly true to type propagules and to ensure micropropagation over along period, to established method to enable twin scale propagation and test the suitable growth regulator and their combination in inducing shoot regeneration and bulbing *in vitro*.

Materials and Methods

Plant material: A commercial grade of onions (cv. Jinhua red), obtained from the farmer was used for study. Bulbs used were often medium in size.

Nutrient media and experimental conditions: The basal nutrient medium consisted of the salt mixture of Murashige and Skoog's formulation. Various concentrations of growth regulators were added to the media, and 3% sucrose was supplemented. The pH of the media was adjusted to 5.6, solidified with 0.7% agar and 0.5% activity charcoal. Then sterilized for 20 min at 1.05 kg/cm^2 pressure. Explants were placed in culture tubes containing 25 ml medium and grown at 22-25°C under Philips fluorescent daylight tubes emitting 3200 lux for 14 h light period. The explants were cultured for a period of three months and observations taken every week. Each treatment level was replicated with fifteen explants.

Preparation of twin scale: To guarantee maximum genetic uniformity, the twin scale used was obtained from undifferentiated meristematic tissue of the bulb. The procedure adopted for bulb preparation was as described below:

Fresh bulb outer scale was carefully removed. The surface layer of the root was cut using a clean sharp knife, to expose clean part. The bulb was then washed in tap water, then washed in distilled water for three times. The explants were dipped in 25% sodium hypochlorite for 20 minutes. After

20 minutes, the bulb was rinsed in distilled water three times at five minutes interval. The bulb was cut longitudinally into two symmetrical parts.
Sodium hypochlorite infiltrated tissue was removed by cutting a thin slice from the cut surface. The remainder of each bulb was cut transversely at its widest point. The lower half was cut into eight equal sectors and from each sector a twin scale' was cut, 10 mm high and 3 mm wide, joined at the base by a small piece of basal plate tissue approximately 1.0 mm high. The above standard procedure of preparing explants from bulb was followed in all experiments.

Shoot production and root induction from twin scale: The twin scale was placed in flask containing 20 25 ml of media containing various kind of growth regulators at different concentrations, and subsequently transferred to the culture room. The experiment was set up with fifteen replicates.
The basal nutrient medium for root formation was supplemented with 3% sucrose, various combinations of NAA and BA, $4PU_{30}$ at different concentrations.

Bulb formation from regenerated shoots: Shoot derived from twin scale was cut at the base culture, then transferred to the basal nutrient media supplemented with various concentrations of $4PU_{30}$. Different concentrations of sucrose on bulbing of regenerated shoot were examined. The explants were cultured for a period of three months. At the end of experiment, the explant was carefully removed from the flask. Using a whatman 42 filter paper, drenching water was imbibed and it was let to stand in dry air for ten minutes. The measurements of the circumference at the largest size were done. The average circumference was recorded. The experiment was set up with fifteen replicates.

Results

Effect of NAA, $4PU_{30}$ and BA on shoot formation in twin scales: The first signs of adventitious shoot formation occurred after one week at all concentrations of NAA. After three weeks, shoot development rate was high in 0.1 mg/l NAA. There was consistence in induction of shoot by NAA (Table 1). The highest shoot percentage from twin scale occurred in 0.1 mg/l NAA after 8 weeks. The lowest shoot induction occurred in 0.05 mg/l NAA. At 0.3 mg/l NAA, shoot formation was 78%. Increasing the concentration to 0.6 mg/l NAA resulted in 80% shoot formation. However, increasing in NAA concentration to 1.0 mg/l did not promote shoot formation tremendously, and only increased it to 84%. The highest number of shoot per explant on twin scale was observed in 0.3 mg/l NAA. The results suggested that the threshold concentration of NAA for inducing shoot from twin scale was between 0.05 and 0.6 mg/l NAA.
According to Duncan's multiple range tests, no significant Difference between the means was observed ($p = 0.05$). Shoot development in NAA + $4PU_{30}$ treatment showed interesting features. Shoot regeneration was vigorous in all explants. Highest rate of shoot regeneration was observed in 0.1 mg/l NAA + 2 mg/l $4PU_{30}$ and regenerated shoot appeared green and healthy at all levels (Table 2). In 0.1 mg/l NAA + 0.5 mg/l $4PU_{30}$, shoot and root formation occurred at the same time. Shoot development in control was similar to that in 0.1 mg/l NAA + 0.5 mg/l $4PU_{30}$. The number of shoot per explant was very high at 0.1 and 2 mg/l $4PU_{30}$ in combination with 0.1 mg/l NAA. Increase in $4PU_{30}$ concentration to 4 mg/l did not improve shoot formation.
Shoot development was comparatively slow in NAA + BA treatment. Prominent shoot initiation became visible at all treatment after two weeks, then poor shoot development occirrred after 4 weeks at high and low concentration of NAA + BA combinations. The highest shoot formation (87%) obseeved was in 0.1 mg/l NAA + 4 mg/l BA followed by 0.1 mg/l NAA + 2 mg/l BA (82 %) (Table 3). The lowest shoot induction of 69% was observed in 0.1 NAA mg/l

Table 1: Effect of NAA on shoots initiated on twin scales

NAA concentration	Shoot formation (%) (mg/l)	Number of shoot per explant
0.00	78	0.89
0.05	70	0.75
0.10	93	0.84
0.30	78	0.91
0.60	80	0.87
1.00	84	0.76

The data represent mean of 15 explants obtained from twin scales

Table 2: Effect of NAA and $4PU_{30}$ on shoots initiated on twin scales

NAA + $4PU_{30}$ concentration	Shoot formation (%)	Number of shoot per plant
0.0	83	0.75
0.1 + 0.1	81	1.13
0.1 + 0.5	83	0.87
0.1 + 1	79	1.25
0.1 + 2	89	1.03
0.1 + 4	78	0.84

The data represent mean of 15 explants obtained from twin scales. According to Duncan's multiple range test, no significant difference between the means was observed ($p = 0.05$)

Table 3: Effect of NAA and BA on shoots initiated on twin scales

NAA + BA concentration (mg/l)	Shoot formation (%)	Number of shoot per plant
0.0	70	0.75
0.1 + 1	69	0.71
0.1 + 2	82	0.78
0.1 + 3	81	0.90
0.1 + 4	87	0.85
0.1 + 5	74	0.84
0.1 + 6	69	0.70

The data represent mean of 15 explants obtained from twin scales. According to Duncan's multiple range test no significant difference between the means was observed ($p = 0.05$)

Table 4: Root formation and the number of root per explant on MS media containing NAA and $4PU_{30}$ after 10 weeks

NAA + $4PU_{30}$ concentration	Root formation (%)	Number of root per explant
0.0	72	0.78
0.1 + 0.1	70	0.69
0.1 + 0.5	84	0.78
0.1 + 1.0	71	1.04
0.1 + 2.0	81	0.95
0.1 + 4.0	75	0.74

Data are mean of roots generated after 10 weeks from 15 explants per treatment According to Duncan's multiple range test, no significant difference between the means was observed ($p = 0.05$)

Table 5: Root formation and the number of root per explant on MS media containing NAA and BA after 10 weeks

NAA + BA concentration (mg/l)	Root formation (%)	Number of root per explant
0.0 + 0.0	71	0.84
0.1 + 1.0	75	1.06
0.1 + 2.0	89	0.67
0.1 + 3.0	84	0.96
0.1 + 4.0	79	1.00
0.1 + 5.0	72	0.86
0.1 + 6.0	70	0.76

Data are mean of roots generated after 10 weeks from 15 explants per treatment. According to Duncan's multiple range test, no significant difference between the means was observed (p = 0.05)

Table 6: Effect of $4PU_{30}$ on bulb formation of cultured shoots after 8 weeks *in vitro*

$4PU_{30}$ concentration (mg/ml)	Bulblets formation (%)	Bulb Circumference (cm)
0	63	1.8
0.1	60	1.6
0.2	66	1.4
0.4	70	2.2
0.8	84	2.6
1.6	78	1.9
3.2	66	1.9

Value is expressed as the mean of the 15 explants

Table 7: Effect of sucrose on bulb formation of cultured shoots after 8 weeks *in vitro*

Sucrose concentration (g/l)	Bulblets formation (%)	Bulb Circumference (cm)
0	33	0.6
25	57	1.0
50	60	1.1
75	75	1.8
100	81	2.9
125	72	1.9
150	70	1.6

About 20 explants per treatment were cultured

+ 1 mg/l BA. There seems to be a relationship between shoot percentage and concentration of BA up to 4 mg/l, however inhibition followed at concentration higher than 4 mg/l.

Effect of NAA, $4P1.1_{30}$ and BA on root formation: Root development from the basal plate of the twin scale was observed after ten days of culturing in 0.1 NAA + 2 mg/l $4PU_{30}$. While, in the rest of the cultures, root development was only observed after three weeks.

A combination of 0.1 mg/l NAA + 0.5 mg/l $4PU_{30}$ resulted in highest root formation from twin scale after ten weeks. The lowest root formation was observed in treatments 0.1 mg/l NAA + 0.1 mg/l $4PU_{30}$ (Table 4).

Root number per explant was not very different among each treatment, and reached a maximum 1.04 at 1.0 mg/1 $4PU_{30}$. With increase in $4PU_{30}$ concentration above 4 mg/l, the root number per explant eventually declined.

In NAA and BA treatment, root formed after two weeks in 0.1 NAA mg/l + 2.0 mg/l BA treatment, a optimal medium. The root appeared thinner and the length of root was short (about 5 mm). When the concentration of BA increased, root formation decreased. Root formation was minimum in treatments 0.1 mg/l NAA + 5 mg/l BA (Table 5). Highest root number per explant was observed at concentration of 0.1 mg/l NAA + 1 mg/l BA. Root number per explant in 2, 3, 5 and 6 mg/l BA was not very different from that of the control.

Effect of $4PU_{30}$ and sucrose on bulb formation: The bulbs first appeared as a swelling at the base of the regenerated shoot after 10 days of culture. Bulb formation of cultured shoots increased with increase in $4PU_{30}$ concentration, 60% in 0.1 mg/l $4PU_{30}$, peaked (84%) at 0.8 mg/l $4PU_{30}$, declined at higher concentration of 3.2 mg/l $4PU_{30}$ (60%).

The circumference of the bulblet ranged between 1.4 and 2.6 cm, it seemed to be related to hormone concentration, with smaller circumference at lower concentrations and increased with the increase in $4PU_{30}$ concentration until 0.8 mg/l declined at 1.6 mg/l. The bulb formed in 1.6 mg/l $4PU_{30}$ more roots compared with other treatment (Table 6).

An increase in sucrose concentration in the culture medium greatly promote the bulblet development, bulblets formation produced on the media ranged within 33-81% , depending on sucrose concentration. A marked increase in the bulblet formation of cultured shoots was observed when the sucrose concentration was increased from 0 to 100 g/l, but decreased at a higher sucrose concentration. Bulblet circumference reached 2.9 cm on a medium with 100 g/l sucrose. This circumference decreased, however, to 0.6 cm in a control medium, and 1.6 cm with a high sucrose concentration of 150 g/l (Table 7).

Discussion

Effect of auxin and cytokinin on shoot regeneration: NAA has been used in inducing shoot regeneration with encouraging success. Normal shoot growth was achieved with 0.1 mg/l NAA (Matsubara and Chen, 1989) . Addition of NAA to the culture medium stimulated Lilium shoot proliferation but also lowered the endogenous auxin content. The number of lilium plantlets produced from intact explant increased with increasing NAA (from 0.5 to 2.0 mμM) (Aartrijk *et al.,* 1982). The concentration of 0.1 mg/l NAA that we have identified compares well with the above observation and concludes that this concentration offers more number of shoots per explant.

The number of regenerated shoots is also associated with BA concentration in the culture medium. Shoots were induced from onion twin scale in media containing 2 to 4 mg/l BA and 0.05 mg/l NAA. Adventitious shoots on leaf, scale and stem explants taken from the basal region of flowering bulbs of onion was induced in 4 mg/l BA and 0.1 mg/l NAA (Hussey, 1982). Our results have also shown that 0.1 mg/l NAA + 4 mg/l BA was the most ideal concentration for inducing shoot in vitro, which is in well agreement with the earlier reports. The effect of NAA + $4PU_{30}$ on shoot formation was also studied in the present experiment. $4PU_{30}$ stimulated shoot formation in toreno, and increased meristem formation in mulberry. Explants taken from seedlings of mulberry (*Mows alba* L.) precultured on 10 mg/l $4PU_{30}$ produced multiple shoots, when 1 mg/l $4PU_{30}$ was used in the pre culture the number of shoots reduced (Ohyama and Oka, 1982). Dramatic increase in shoot number in hardy deciduous azaleas (Rhododendron sp.) cultured in 0.5 m M $4PU_{30}$ medium (Fellman *et al.,* 1987). In our experiment observed that the concentration of 2 mg/l $4PU_{30}$ to be the most effective in inducing onion shoot from twin scale.

$4PU_{30}$ were more effective than BA for cell development and growth in tobacco (Okamoto *et al*., 1982). From our experiment we also found that $4PU_{30}$ was more effective than BA in inducing shoot regeneration.

Root formation: Induction of shoots or roots was dependent on the cytokinin/auxin combination (Ramirez-Malagon and Ochoa-Alejo, 1991). An extensive hairy root production follows shoot regeneration in medium containing a low auxin/cytokinin balance (Delpierre and Boccon-Gibod, 1992). Media lacking BA led to the formation of progressively more roots with numerous root hairs as NAA concentrations increased.

From our results, we found there is a decrease in root development with the presence of BA, this agrees with the findings of others. It is speculated this could be due to inefficient hormone use by the target cells of explant. Root percent did not increase greatly with the increase in concentration of $4PU_{30}$, which could be due to a little effect of $4PU_{30}$ on root formation. Interestingly, the twin scale produced red color with $4PU_{30}$ after 10 days, which we suspect to be due to promotion of the synthesis of pigment of onion by $4PU_{30}$.

Bulb formation: Bulb formation was proportional to the sorts and concentrations of growth regulators in media. The formation of bulb confirms the influence of $4PU_{30}$ in inducing bulb development in vitro. Average circumference of the resultant swellings varied with the concentration of $4PU_{30}$. At lower concentrations circumference of swelling was small and progressed through 0.8 mg/l $4PU_{30}$ and started to decline. The largest circumference obtained in 0.8 to 1.6 mg/l $4PU_{30}$ suggests that is the suitable concentration for bulb formation. A range of sucrose concentration has been investigated in inducing bulb formation in onion. The range of sucrose concentration at which bulb starts to form is critical. A concentration of around 30 to 120 g/l sucrose had greater critical effect.

The capability of sucrose to inhibit shoot development results in increase in basal swelling. This mechanism is important because it ensures that no effective aerial part remains, which may otherwise compromise genuine storage organ formation.

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