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*Full Length Research Paper*

# Effect of culture conditions on the plant regeneration *via* organogenesis from cotyledonary node of cowpea (*Vigna unguiculata* L. Walp)

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**A rapid and efficient regeneration system *via* organogenesis from cotyledonary node explants of cowpea (*Vigna unguiculata* L. Walp) has been established. The cotyledonary node explants excised from 4 days old seedlings, placed *in vitro* on medium containing salts of Murashige and Skoog and vitamins of Gamborg's media ($MSB_5$). Adventitious shoots occurred at the basal end of the initiated axillary buds that preexisted at the node regions. BAP at 1.25 mg/l was the optimum for shoot induction. The combination of BAP with IBA had worthless effect on shoots proliferation. The number of adventitious buds was promoted when the seeds were preconditioned with appropriate concentrations of BAP (2 to 3 mg/l), whereas was depressed with higher concentrations of BAP (5 to 15 mg/l). The regeneration system was further optimized due to the presence of cotyledons attaching to the cotyledonary node explants. Explants with two entire cotyledons from 4 days old seedlings produced the greater number of shoots (7.83) after 3 weeks on $MSB_5$ medium supplemented with 1.25 mg/l BAP. Regenerated shoots could well elongate on regulator-free basal medium and well root with 100% of success on the half strength medium supplemented with various concentrations of IBA (0, 0.1, 0.3, and 0.5 mg/l). The regenerated plantlets were cultured on the pots containing sterilized vermiculite and soil (1:1) with 27% of survival.**

**Key words: Cowpea,** plant regeneration, cotyledonary node, organogenesis.

## INTRODUCTION

Large-seeded legume cowpea (*Vigna unguiculata L.* Walp) is favored all over the world for its high protein content in seeds and is a major source of dietary protein of poor farmers in Sub-Saharan Africa and Asia (Sahoo et al., 2003). It is also used as animal fodder, cover crop, green manure and nitrogen-fixing crop (Aasim et al., 2009a). Despite its significant nutritional value, the grain productivity is seriously affected and the crop suffers little progress for the strong hybrid-incompatibility (Singh heavy losses primarily due to its prominent susceptibility to insect pests and pathogens (Sahoo et al., 2001). There are 31 insect species identified in the cowpea, among the most damaging insects, aphids, flower thrips, cowpea pod borer, pod sucking bugs, the cowpea weevil, and the leaf beetle are the major pre-harvest entomological pests. Insect can cause 20 to 60% losses in yield. Fungal diseases are the second constraints in cowpea cultivation. Out of these, anthracnose, brown blotch, leaf spots, web blight are prominent (Latunde, 1990; Kormawa et al., 2000). In spite of some resistance genes to insect pests and fungi have been identified in some closely related Vigna species (such as *V. vexillata*) (Latunde, 1990; Ortiz, 2003), the attempts by using the conventional breeding methods to introduce the resistance genes into the cultivated cowpea have made et al., 1997). The identification of candidate genes for insect pest resistance in cowpea (including *Bacillus*

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**Abbreviations: BAP,** Benzylaminopurine; **IBA,** indole-3-butyric acid.

*thuringenesis* protoxin genes, and genes coding α-amylase inhibitor, protease inhibitor and lectins) provided some opportunities for biotechnology in cowpea (Singh et al., 2000). Hence, the transfer of insect resistance genes to cowpea, by genetic transformation, has the potential to address some of these problems and could have a major impact on food security on the African continent (Machuka, 2000; Zaidi et al., 2005).

A reliable plant regeneration protocol is prerequisite for genetic transformation. Plant regeneration of cowpea via organogenesis has been achieved from primary leaves, epicotyls, hypocotyls, cotyledons, cotyledonary node, shoot meristem, shoot tip, plumular apices (Muthukumar et al., 1995; Bao et al., 2006; Amitha and Reddy, 1996; Pellegrineschi, 1997; Raveendar et al., 2009; Manoharmin et al., 2008; Aasim et al., 2009a,b). Among those explants, the easy-to-culture cotyledonary node explants seemed the most responsive for the induction of multiple shoots, which was appropriate to agrobacterium-mediated transformation (Chaudhury et al., 2007; Raji et al., 2008; Solleti et al., 2008; Adesoye et al., 2010).

Some achievements have been gained in abroad. However, there were only two reports about the regeneration of cowpea in China and the frequency of regeneration was very low (Li et al., 1993; Bao et al., 2006).

In this paper, we studied the culture factors that are responsible for plant regeneration from cotyledonary node with a Chinese cowpea cultivar for the first time. It is aiming to use the results in genetic transformation researches in future.

## MATERIALS AND METHODS

### Plant material and explant preparation

Mature seeds of *cv.* Cheng-jiang Vll of cowpea were obtained from the Research Institute of Horticulture, Academy of Chengdu Agriculture and Forestry Science, Chengdu, China. The uniform and healthy seeds were surface sterilized with 70% ethanol for 1 min, followed by a soak with 0.2% (w/v) $HgCl_2$ for 5 min, and finally rinsed five times with sterile distilled water and blotted with sterilized filter papers. The sterilized seeds were germinated on basal medium for 4 days. The cotyledonary node explants were excised by removing both the cotyledons and cutting both the epicotyls and hypocotyls approximately 2 mm above and below the node region.

### Culture media and conditions

The basal media $MSB_5$ containing MS salts (Murashige and Skoog, 1962), $B_5$ (Gamborg et al., 1968) vitamins, 3% (w/v) sucrose and 0.7% agar was used in all experiments. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121°C for 20 min. The cultures were maintained at 25 ± 2°C in an incubator under a 16 h photoperiod using white fluorescent tubes.

### Shoot inducing

The cotyledonary node explants were cultured on $MSB_5$ medium containing various concentrations of BAP (0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 mg/l) and IBA (0 and 0.1 mg/l) to assess the influence of growth regulators on multiple shoot buds induction. The regenerated shoots were transferred to fresh medium every 1 week.

### Seeds preconditioning

To study the effect of seeds preconditioning with higher concentration of cytokinin on shoot regeneration, the seeds were placed on the germination medium supplemented with different concentrations of BAP (0, 2, 3, 4, and 5 mg/l). Then the excised cotyledonary node explants were cultured in the regeneration medium with the optimal BAP and IBA concentrations that had been selected before.

### Explants optimization

The regeneration capacity of cotyledonary node explants with one cotyledon and two cotyledons were comparing with the explants without cotyledon, which was to demonstrate the effect of the existence of cotyledon on shoot buds reproduction. All the explants were cultured on the medium with the optimal growth regulator selected before.

### Shoot elongation, rooting and plantlets hardening

After 3 weeks of induction cultures, the axillary buds were removed and the multiple shoot buds were transferred to the regulator-free medium $MSB_5$ for shoot elongation for 1 week, and then the elongated shoots were cultured on half strength $MSB_5$ medium supplemented with various concentrations of IBA (0, 0.1, 0.3, and 0.5 mg/l) for rooting. After 2 weeks of culture, the frequency of rooting rate was recorded. The plantlets with well-developed roots were removed from the culture medium and after washing the roots with tap water, the plantlets were transferred to pots containing sterilized vermiculite: soil (1:1) and covered with polybags to ensure high humidity during the first days. Potted plantlets were placed in culture room with the same condition during regeneration. Subsequently the plants were transferred to greenhouse.

### Statistical analysis

After 3 weeks of culture, the frequency of regeneration and the average number of shoots per explant were recorded. All experiments were completely randomized and repeated three times with 20 explants per treatment. The data for the frequency of regeneration, the average number of shoots per explant, and the percentage of rooting were subjected to analysis of variance and significant treatment differences using Duncan's new multiple range method with the help of statistical software “DPS6.5 for Windows” (Tang and Feng, 2002).

## RESULTS AND DISCUSSION

### Effect of different concentrations of BAP-IBA

Since BAP alone or in combination with lower auxins is one of the most effective and widely used protocols for shoot induction in numerous leguminous plants (Franklin et al., 2000; Nagori and Purohit, 2004; Koné et al., 2009; Aasim et al., 2009a), we have tested this plant growth

**Table 1.** Effect of different concentrations of BAP-IBA on shoot regeneration response from cotyledonary node explants of cowpea for 3 weeks.

| Plant hormones (mg/l) | | Frequency of axillary buds regeneration (%) | Frequency of adventitious shoots regeneration (%) | Mean number of shoots per explants (including axillary buds) |
|---|---|---|---|---|
| BAP | IBA | | | |
| 0.00 | 0.00 | 76.67 | 3.34 | 1.54$^{e}$ |
| 0.25 | 0.00 | 86.67 | 23.33 | 1.80$^{e}$ |
| 0.50 | 0.00 | 100.0 | 56.70 | 2.28$^{d}$ |
| 0.75 | 0.00 | 96.65 | 80.00 | 3.07$^{c}$ |
| 1.00 | 0.00 | 100.0 | 96.56 | 3.93$^{b}$ |
| 1.25 | 0.00 | 100.0 | 100.0 | 4.47$^{a}$ |
| 1.50 | 0.00 | 100.0 | 100.0 | 4.20$^{ab}$ |
| 2.00 | 0.00 | 100.0 | 86.49 | 2.83$^{c}$ |
| 0.00 | 0.10 | 80.00 | 6.68 | 1.60$^{e}$ |
| 0.25 | 0.10 | 100.0 | 26.64 | 1.91$^{e}$ |
| 0.50 | 0.10 | 100.0 | 63.32 | 2.40$^{d}$ |
| 0.75 | 0.10 | 100.0 | 100.0 | 2.90$^{c}$ |
| 1.00 | 0.10 | 100.0 | 100.0 | 3.17$^{c}$ |
| 1.25 | 0.10 | 100.0 | 100.0 | 4.33$^{a}$ |
| 1.50 | 0.10 | 100.0 | 96.67 | 3.90$^{b}$ |
| 2.00 | 0.10 | 100.0 | 83.50 | 2.77$^{c}$ |

regulator combined with IBA at various concentrations (Table 1). The cotyledonary node explants were excised and vertically cultured on $MSB_5$ medium. After 3 to 4 days of culture, the cotyledonary node explants began to expand and form calluses from the embedded ends of the explants. The axillary buds started to initiate at the node regions and well developed after 1 week of culture. The frequency of axillary buds regeneration was very high (76.67 to 100.0%) and the multiple shoot buds began to emerge around the basal ends of well developed axillary buds. Multiple shoot buds proliferation was favored in presence of BAP. The combination of BAP (0.00 to 2.00 mg/l) with or without IBA (0.10 mg/l) had similar effects on multiple shoot buds production (Table 1). This revealed that adding IBA to the medium had a worthless effect on shoot buds formation, which was agreed with Aasim et al. (2009a). The frequency of multiple shoot buds regeneration and the mean number of shoots per explants increased with the increasing of the concentrations of BAP (0.00 to 0.75 mg/l). BAP at 1.25 or 1.5 mg/l was optimal for shoot induction which could produce an average of over 4 shoots in all treatments (Figure 1a). Increasing BAP concentration to 2 mg/l reduced shoot proliferation and increased the differentiation of abnormal shoots and suppressed the elongation of the shoots. The explants cultured on medium without plant hormones could only produce axillary buds and the axillary buds could easily induce roots on subculture medium to form full plantlets. The length of axillary buds and multiple shoot buds declined with the increasing of BAP concentrations (data not show), which agreed with the pioneering researches (Diallo et al., 2008; Aasim et al., 2009a). Since maximum frequency and number of shoots were observed on the medium containing 1.25 mg/l BAP, hence this concentration was selected to further studies.

## Effect of seed preconditioning

Some pioneering studies revealed that preconditioning of seeds on the germination medium with higher concentration of cytokinins had positive effect for later shoot induction (Brar et al., 1999; Van Le et al., 2002; Manoharmin et al., 2008; Solleti et al., 2008; Raveendar et al., 2009). To verify this, the seeds were pretreated with different concentrations of BAP to germinate (Table 2). Comparing with the control, the germinated seedlings cultured on the pretreated medium were obviously stronger, with larger cotyledons, thicker and shorter hypocotyls and the end of the stems expanded, which was convenient for the cutting of the explants (Figure 1b). After removal of both the cotyledons and both the epicotyls and hypocotyls from the seedlings, the well excised cotyledonary node explants were placed vertically onto MSB5 medium supplemented with 1.25 mg/l BAP. After 3 to 4 days of culture, the explants began to expand and elongate from the embedded ends and initiated axillary buds at the node region. Comparing with the control, the embedded ends of the explants detached from the preconditioned seedlings formed less calluses or without any calluses. Concerning on the frequency of axillary buds regeneration, there was no significant difference among different treatments. Contrary to the control, the axillary buds induced from the pretreated explants were conspicuously shorter, with larger leaflets. Some of those axillary buds were hard to elongate during

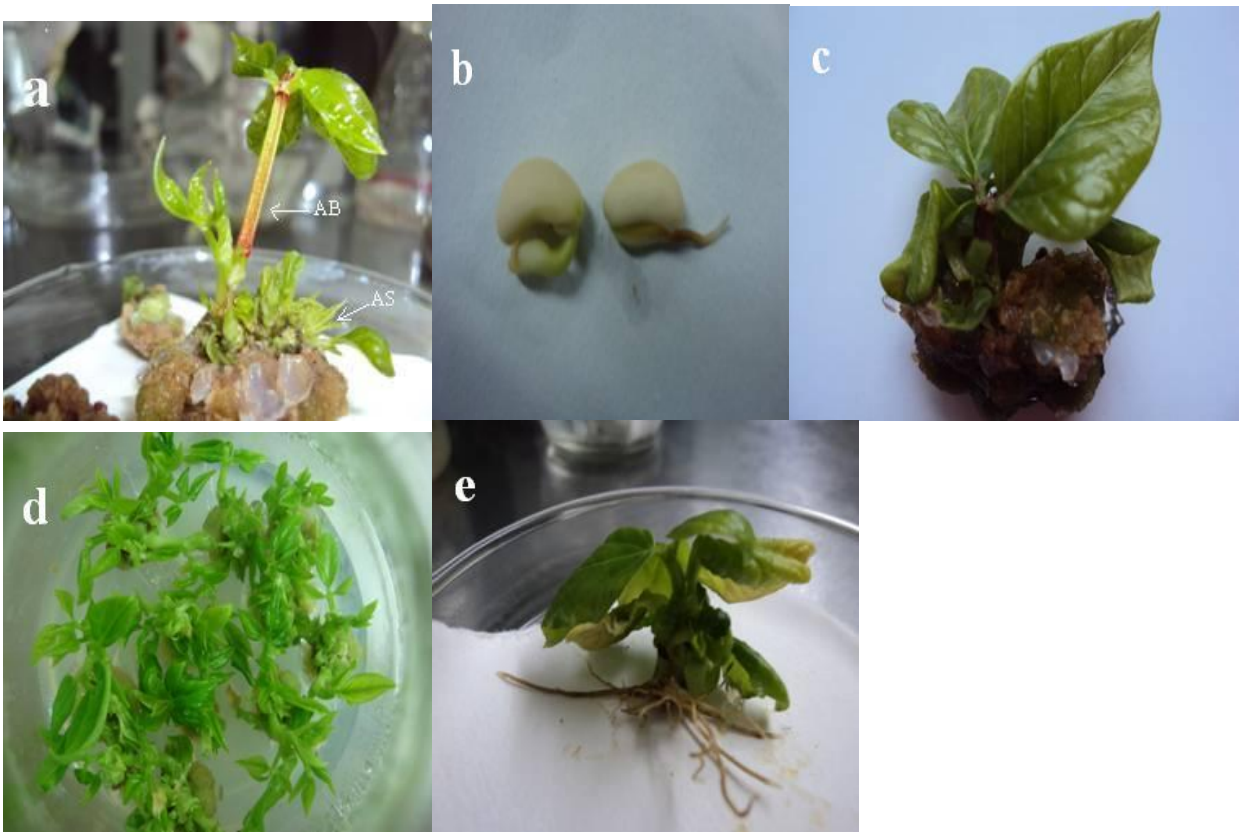


**Figure 1.** Organogenesis system of cowpea. (a) Adventitious shoots initiated from the basal end of axillary buds in 2 weeks of culture (AS was adventitious shoots, AB was axillary buds initiated from preexistent meristem). (b) Seedling preconditioning with 3mg/l BAP (left) and seedling without preconditioning (right). (c) Adventitious shoots initiated from the basal end of axillary buds in 2 weeks of culture (The explant isolated from the seedlings preconditioned with15 mg/l BAP). (d) Adventitious shoots initiated from the cotyledonary node explants attached with two cotyledons for 3 weeks of culture (two cotyledons were removed). (e) Induction of roots from in vitro regenerated shoots cultured on half strength $MSB_5$ medium supplemented with 0.5mg/l IBA for 2 weeks of culture.

the subcultures when the preconditioning concentrations of BAP were up to 5 mg/l. Pretreatment the seedlings with lower concentrations of BAP (2 to 4 mg/l) improved the number of adventitious shoots, but the number of adventitious shoots decreased when the preconditioning BAP concentrations were up to 5 mg/l (Table 2). There were only some fasciated leaflets regenerated around the end of axillary buds and those leaflets could not develop into normal shoots when the preconditioning concentrations of BAP were up to 5 mg/l. (Figure 1c). Hence the pretreatment of seeds during germination on medium supplemented with lower concentrations of BAP are favored for the multiplication of shoots and with higher concentrations of BAP inhibited.

## Effect of the presence of cotyledon

Some previous studies asserted that the presence of cotyledons on cotyledonary node explants improved the regeneration frequency (Gassama, 1996; Diallo et al., 2008). To further optimize the regeneration system, the explants with single or both the cotyledons were used (Table 3). After 2 to 3 days of culture, the attached cotyledons began to turn green and the axillary buds started to initiate from the node regions. After 1 week of culture, we found that the frequency of axillary buds regeneration and the number and length of axillary buds were promoted by the existence of cotyledons. Except for the control, all of the treatments could produce two axillary buds in all cultures (Table 3). With the development of axillary buds, the adventitious buds initiated from the basal ends of axillary buds. The number of multiple shoots regeneration was significantly affected by the presence of cotyledons. But there was no obvious difference between the explants with single cotyledon and the explants with both cotyledons, the number of shoots per explant could approximately obtain eight. (Figure 1d). Because of the cotyledons began to wrinkle and etiolate, they were removed after 2 weeks of culture. The presence of cotyledons enhanced the number of shoots not only due to the effect of nutritious reserve accumulated

**Table 2.** Effect of preconditioning with different concentrations of BAP on shoot regeneration response from cotyledonary node explants of cowpea for 3 weeks.

| Preconditioning concentrations of BAP (mg/l) | Frequency of axillary buds regeneration (%) | Frequency of multiple shoot buds regeneration (%) | Mean number of shoots per explants (including axillary buds) |
|---|---|---|---|
| 0 | 100 | 100 | $4.47^{bc}$ |
| 2 | 100 | 100 | $4.9^{ab}$ |
| 3 | 100 | 100 | $5.33^{a}$ |
| 4 | 100 | 100 | $4.27^{c}$ |
| 5 | 100 | 100 | $3.93^{c}$ |
| 10 | 100 | 93.3 | $2.91^{d}$ |
| 15 | 100 | 80 | $2.43^{d}$ |

**Table 3.** Effect of the attachment of cotyledons on shoot regeneration response from cotyledonary node explants of cowpea for 3 weeks.

| Explant type | Frequency of axillary buds regeneration (%) | Frequency of multiple shoot buds regeneration (%) | Mean number of shoots per explants (including axillary buds) |
|---|---|---|---|
| Cotyledonary node without both the cotyledons | 100 | 100 | $4.47^{b}$ |
| Cotyledonary node with one cotyledon | 100 | 100 | $7.70^{a}$ |
| Cotyledonary node with both the cotyledons | 100 | 100 | $7.83^{a}$ |

in cotyledons and ready available for plantlet growth, but also due to the reduction of wounds that decreased the browning of explants.

After 3 weeks of shoot induction culture, bud clusters of differentiated shoots were separated and grown individually on basal hormone-free MSB5 medium to elongate. Then the elongated shoots were transferred to half strength $MSB_5$ medium supplemented with various concentrations of IBA (0, 0.1, 0.3, and 0.5 mg/l) for rooting. The results indicated that no significantly difference was noticed in terms of percentage of rooting, which all of treatments could root well in the rooting medium (Figure 1e). The well developed plantlets were cultured on the pots containing sterilized vermiculite and soil (1:1) with 52% of survival (data not shown). The protocol described here revealed simple but high *in vitro* morphogenetic responses of cowpea. The system is also rapid, requiring only 7 weeks from induction of shoots to transformation of plantlets to soil. It is useful for later gene transformation study.

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