

## Comparative studies on the competence of axillary shoot regeneration on unsliced and longitudinally sliced cotyledon nodes of *Vigna unguiculata*

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**Abstract:** *Vigna unguiculata* is an important food legume crop in the semiarid tropics. It suffers from a host of agricultural constraints including damage due to diseases and pests. Judicious application of biotechnological methods can lead to considerable improvement in this important crop. Shoot regeneration from unsliced and longitudinally sliced cotyledon node explants obtained from 3-day-old to 5-day-old seedlings grown in vitro was achieved on Murashige and Skoog (MS) medium containing 1.11, 2.22, 3.33, and 4.44  $\mu\text{M}$  benzylaminopurine (BA) supplemented with 1.0 mg/L polyvinylpyrrolidone and 500 mg/L bacteriostatic Augmentin. Callus induction was recorded in all culture media on both explants. The shoot regeneration frequency (%) of longitudinally sliced cotyledon node explants was 2-fold to 3-fold higher than that of unsliced cotyledon node explants. A maximum number of 9.92 shoots per longitudinally sliced cotyledon node explant was recorded on MS medium containing 3.33  $\mu\text{M}$  BA. Each increase in the BA concentration of the culture medium proportionally decreased the mean shoot length on both explants. Maximum mean shoot lengths of 2.80 cm on unsliced cotyledon nodes and 3.04 cm on longitudinally sliced cotyledon node explant were recorded on MS medium containing 1.11  $\mu\text{M}$  BA. Regenerated shoots were rooted on MS rooting medium containing 2.45  $\mu\text{M}$  indole-3-butyric acid. In vitro regenerated plants were acclimatised at room temperature in growth rooms, where they produced viable seeds.

**Key words:** Auxin, cytokinin, in vitro, legume, micropropagation

### *Vigna unguiculata* bitkisinde dilimlenmemiş ve dikey dilimlenmiş kotiledon boğum eksplantların sürgün rejenerasyonuna etkileri

**Özet:** Börülce yarı kurak tropik bölgelerin önemli yemeklik tane baklagil bitkisi olup, bazı zararlara maruz kalmaktadır. Bu bitkinin ıslahı için biyoteknolojik yöntemlerin geliştirilmesi son derecede önemlidir. Bu çalışmada, in vitro koşullarda 3-5 günlük çimlenmiş fideliklerden elde edilen dilimlenmemiş ve dikey dilimlenmiş kotiledon boğum eksplantları 1,11, 2,22, 3,33 ve 4,44  $\mu\text{M}$  benzylaminopurine (BA), 1,0 mg/L PVP ve 500 mg/L Augmentin içeren MS ortamda kültüre alınarak sürgün rejenerasyonu elde edilmiştir. Tüm ortamlarda her iki eksplant üzerinde de kallus oluşumu gözlenmiştir. Dikey dilimlenmiş kotiledon boğum eksplantlarında dilimlenmemiş kotiledon boğum eksplantlarına göre daha fazla sürgün oluşum görülmüştür. Dilimlenmiş kotiledon boğumunda, en fazla sürgün (9,92 adet) 3,33  $\mu\text{M}$  BA içeren MS ortamından elde edilmiştir. Tüm kültür ortamlarında, BA dozların artışı oransal olarak sürgün uzunluğunun azalmasının sebebi olmuştur. En uzun sürgün oluşumu (2,80 cm ve 3,04 cm), dilimlenmiş ve dilimlenmemiş kotiledon boğumundan 1,11  $\mu\text{M}$  BA içeren MS ortamında görülmüştür. Elde edilen sürgünler 2,45  $\mu\text{M}$  IBA içeren MS ortamda köklendirilmiştir. In vitro koşullarda gelişen bitkilerin adaptasyonu oda sıcaklığında sağlanmış olup, tohum elde edilmiştir.

**Anahtar sözcükler:** Oksin, sitokinin, in vitro, baklagil, mikroçoğaltım

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## Introduction

Cowpea is one of the most important food legume crops in the semiarid tropics covering Asia, Africa, southern Europe, and Central and South America. Cowpea is a drought-tolerant, warm-weather crop that has the ability to fix atmospheric nitrogen through root nodules. Cowpea is shade-tolerant and is also used as an intercrop with cotton (Aasim et al., 2008b), maize (Van Kessel & Roskoski, 1998), millet (Ntare, 1990), and sorghum (Khan et al., 2000).

Cotyledon node is reported to be an important explant for genetic transformation in lentil (Khawar & Ozcan, 2002; Dogan et al., 2005; Sevimay et al., 2005), narbon vetch (Kendir et al., 2008, 2009), Hungarian vetch (Sahin-Demirbag et al., 2008), chickpea (Sanyal et al., 2003), and pea (*Pisum sativum* L.) (Svabova et al., 2005). There was one report each on shoot regeneration from unsliced cotyledon nodes of *Vigna radiata* L. (Gulati & Jaiwal, 1994) and Asiatic *Vigna* species (Avenido & Hattori, 1999) and a few reports on shoot regeneration from the unsliced cotyledon node of *Vigna unguiculata* (L.) Walp. (Van Le et al., 2002; Popelka et al., 2006; Chaudhury et al., 2007; Diallo et al., 2008). However, there have not been any reports on shoot regeneration from longitudinally sliced cotyledon node explants of cowpea.

This study aimed to compare the in vitro shoot regeneration of unsliced cotyledon node and longitudinally sliced cotyledon node explants of Turkish cowpea cultivar Akkiz on Murashige and Skoog (MS) medium containing variants of benzylaminopurine (BA).

## Materials and methods

Seeds of Turkish cowpea cultivar Akkiz were obtained from the Department of Field Crops of the Faculty of Agriculture of Ege University in İzmir, Turkey. The seeds were surface sterilised with 70% commercial bleach (Ace<sup>®</sup>) containing 5% NaOCl for 5 min, followed 3 times by 5 min of rinsing with bidistilled sterilised water, and were cultured for germination on 35 mL of MS basal medium (Murashige & Skoog, 1962). MS medium was contained in 100 × 10 mm petri dishes and supplemented with 3.0% sucrose. Agar (0.65%, Duchefa) was added to the culture

medium after adjusting the pH to 5.6-5.8 before autoclaving at 121 °C for 20 min. Unsliced cotyledon node explants (with 2 buds) and longitudinally sliced cotyledon node explants (containing only a single bud) were excised from 3- to 4-day-old seedlings grown in vitro (Figure 1). They were cultured on 35 mL of MS medium containing 0 (control), 1.11, 2.22, 3.33, or 4.44 µM 6-benzylaminopurine (BA) supplemented with 1 mg/L polyvinylpyrrolidone (PVP), 3.0% sucrose gelled with 0.65% agar, and 500 mg/L Augmentin, an antibiotic used exclusively to eradicate bacterial contaminations. The pH of all media was adjusted to 5.6-5.8 using 0.1 N KOH or 0.1 N HCl before sterilisation and solidified with 0.65% agar. All cultures were incubated in growth chambers at 24 ± 2 °C with a 16-h light photoperiod.

After 6 weeks of regeneration, well-developed shoots obtained from unsliced cotyledon node explants regenerated on MS medium containing 2.22 or 3.33 µM BA were excised under aseptic conditions and were rooted on 35 mL of MS medium containing 2.45 µM indole-3-butyric acid (IBA) in Magenta GA7 vessels. After 4 weeks, agar was carefully removed from the roots by washing them under tap water, followed by submerging the rooted plants in water for 15-20 min to maintain turgor and avoid wilting. They were transferred to pots containing vermiculite, organic matter, and sand (1:2:1). These pots were then transferred to the greenhouse at room temperature, where they were subjected to an intermittent mist-water spray by an HR-15 Cool Mist

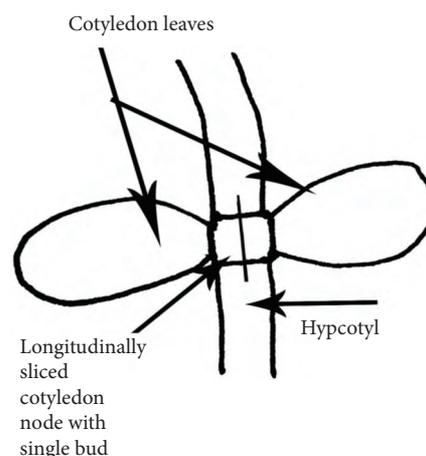


Figure 1. Schematic drawing of a longitudinally sliced cotyledon node with single bud.

humidifier with the humidistat turned on for 24 h. Relative humidity was maintained at 90% during the first few days, which helped to maintain a film of water on the plant leaves in order to avoid wilting. The humidity was gradually reduced to 40% in 2 weeks.

All treatments of regeneration and rooting experiments had 6 replicates containing 5 explants each ( $6 \times 5 = 30$  explants). Data on the frequency (%) of shoot regeneration, mean number of shoots per explant, shoot length, and frequency of rooting was recorded and analysed using one-way ANOVA with SPSS 16 for Windows. The post hoc tests were performed using Duncan's multiple range test. Data given in percentages were subjected to arcsine transformation (Snedecor & Cochran, 1989) before statistical analysis.

## Results

Initial experiments in the laboratory showed that the seeds bore a large number of endogenic bacteria and secreted a large number of phenolic compounds, both of which created problems in culturing the explants and subsequent regeneration. The bacterial contaminants could not be eradicated using commercial bleach. Therefore, endogenic bacterial contaminants were eliminated by adding 500 mg/L Augmentin (a broad spectrum bactericide from GlaxoSmithKline) to the seed germination and regeneration media after autoclaving at 40-45 °C, before pouring the media into petri dishes or Magenta

GA7 vessels. The problem of phenolic compounds was avoided by supplementing the regeneration medium with 1 mg/L PVP.

Axillary shoot regeneration was recorded in all culture media irrespective of the type of explants. Both explants swelled and produced morphogenic calli at the wounds in contact with the regeneration medium. Shoot meristems were visible after a week on unsliced cotyledon node explants on MS medium containing different concentrations of BA and after 4-5 days on longitudinally sliced cotyledon node explants; shoots were visible after the second week of culture (Figure 2). No shoot regeneration was recorded on either of the explants on MS medium without growth regulators (control). Analysis of variance showed that the frequency of shoot regeneration, number of shoots per explant, and shoot length from unsliced and longitudinally sliced cotyledon node explants were all significantly ( $P < 0.05$ ) affected by the concentration of BA in the MS regeneration media. Comparing the shoot regeneration frequency of unsliced (8.33%-41.67%) and longitudinally sliced cotyledon nodes (75.00%-83.33%), sharp variations between the 2 nodes (Table) were very evident. Each increase in the concentration of BA in the culture medium resulted in a proportional increase in the mean number of shoots per explant in longitudinally sliced cotyledon nodes. Maximum numbers of 9.33 shoots per unsliced cotyledon node and 9.92 shoots per longitudinally sliced cotyledon node were recorded on MS medium containing 2.22 and 4.44  $\mu\text{M}$  BA, respectively. During the initial stages of

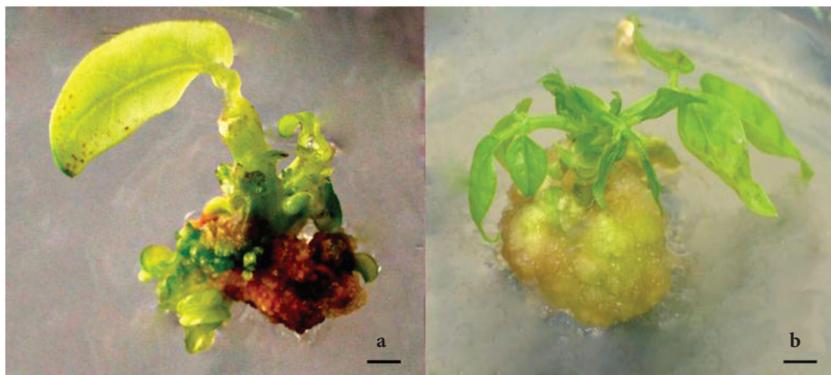


Figure 2. Initiation of well-defined shoots on cultivar Akkiz using a- an unsliced cotyledon node and b- a longitudinally sliced cotyledon explant after the second week of culture. Scale bar = 0.23 cm.

Table. Effects of MS media containing variants of BA on shoot regeneration from unsliced cotyledon nodes and longitudinally sliced cotyledon node explants from cowpea cultivar Akkiz.

Explant type	BA ( $\mu\text{M}$ )	Number of explants	Frequency (%) of shoot regeneration	Mean number of shoots per explant	Mean shoot length (cm)
Unsliced cotyledon node	1.11	30	25.00 <sup>b</sup>	6.00 <sup>c</sup>	2.80 <sup>a</sup>
	2.22	30	25.00 <sup>b</sup>	9.33 <sup>a</sup>	2.40 <sup>b</sup>
	3.33	30	38.33 <sup>c</sup>	8.00 <sup>b</sup>	2.10 <sup>bc</sup>
	4.44	30	41.67 <sup>a</sup>	6.50 <sup>c</sup>	2.00 <sup>c</sup>
Longitudinally sliced cotyledon node	1.11	30	75.00	3.20 <sup>c</sup>	3.04 <sup>a</sup>
	2.22	30	75.00	6.33 <sup>b</sup>	1.61 <sup>b</sup>
	3.33	30	83.33	9.92 <sup>a</sup>	1.10 <sup>c</sup>
	4.44	30	83.33	9.79 <sup>a</sup>	1.08 <sup>c</sup>

Values within a column in a block followed by different small letters are significantly different at the 0.05 level according to Duncan's test.

development, multiple twisted shoots were recorded on unsliced cotyledon nodes (Figure 3); these shoots untwisted during later stages of growth. No twisting of shoots was observed in the longitudinally sliced cotyledon node explants during any stage of growth (Figure 3).

Each increase in the BA concentration of the MS media had a negative effect on the mean shoot length in both explants. Mean shoot length in unsliced cotyledon nodes and longitudinally sliced cotyledon nodes ranged from 2.00 to 2.80 cm and from 1.08 to

3.04 cm, respectively. A maximum mean shoot length of 3.04 cm was recorded in longitudinally sliced cotyledon node explants on MS medium containing 1.11  $\mu\text{M}$  BA.

In a 4-week period, the regenerated shoots from both explants rooted easily on MS medium containing 2.45  $\mu\text{M}$  IBA in Magenta vessels. All plants easily acclimatised in the greenhouse, survived, and grew very well. The first flower buds were recorded after 45 days (Figure 3). All acclimatised plants set seeds in 60-75 days.

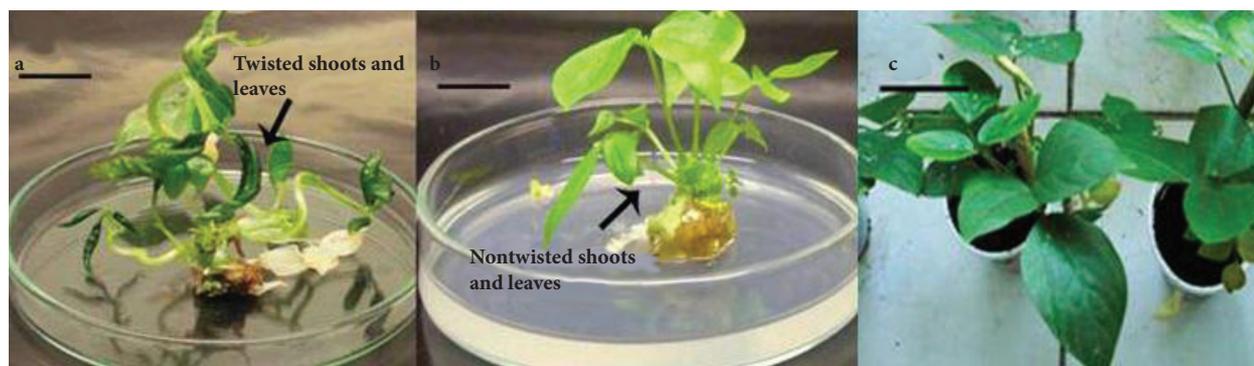


Figure 3. Shoot regeneration from cotyledon node and longitudinally sliced cotyledon node explant of cowpea cultivar Akkiz: a- regeneration of multiple twisted shoots and leaves on the unsliced cotyledon node, b- multiple uniform and nontwisted shoot regeneration on the longitudinally sliced cotyledon node explant, c- acclimatisation in the green house. Scale bars: a and b = 1.30 cm, c = 5 cm.

## Discussion

Callus induction was observed at the proximal ends on both explants irrespective of the concentration of BA in the medium, and all of the BA concentrations were found equally effective for the production of calli. These results are in agreement with those of Aasim et al. (2008a), who reported callusing followed by multiple shoots from the shoot meristems of 3-day-old to 5-day-old *in vitro*-grown seedlings of Turkish cowpea cultivar Akkiz on cultures containing 0.5 mg/L (2.22  $\mu$ M) BA with and without 1-naphthaleneacetic acid (NAA). The results are also in agreement with previous studies that confirm that, on rare occasions, cytokinins have also induced calli from leaves, cotyledon, shoot apices, and hypocotyl in sunflower (Greco et al., 1984), leaf explants of *Coffea arabica* L. (Yasuda, 1985), leaf explants of sugar beet (Masuda et al., 1988), and cotyledon nodes with 1 or 2 cotyledons of cowpea (Diallo et al., 2008).

Cotyledonary nodes from mature seeds with or without cotyledons have proven to be most responsive for the induction of multiple shoots and generate transgenic plants in grain legumes like soybean (Olhoft et al., 2003), pea (Bean et al., 1997), pigeon pea (Geetha et al., 1999), mungbean (Jaiwal et al., 2001), black gram (Saini et al., 2003), and cowpea (Van Le et al., 2002). However, there have been no reports of the use of longitudinally sliced cotyledon nodes containing single buds in any of the previous studies. The regeneration response from the cotyledonary nodes may be comparatively higher than that of other explants. The junction of the cotyledon and the embryo axes contain axillary meristematic cells that are highly regenerable. The regenerative potential of cell types from unsliced cotyledon nodes with 2 buds and longitudinally sliced cotyledon nodes with 1 bud was highly variable in terms of the shoot regeneration frequency (%), mean number of shoots per explant, and mean shoot length. Induction of the regenerative process in such tissues depends on the competence of the cells for reprogramming genetic patterns. BA has been reported to activate the presence of totipotent cells at the cotyledonary region, leading to shoot formation after *in vitro* culture (Cheng et al., 1980; Wright et al., 1986). Avenido and Hattori (2001) reported the cotyledon stem junction or the area just above the detachment area to be regeneration sites once the cotyledons are removed. Avenido and

Hattori (1999) found that the axillary buds in the cotyledon node are fully developed in the epigeal region of mungbean (*Vigna radiata* L.) and in the hypogeal region of allotetraploid *Vigna glabrescens* (4 days after germination) in medium containing MS salts and B5 vitamins; this led them to conclude that the capacity of explants to regenerate axillary shoots was already predetermined at the time of culture and that their full potential could be seen in the presence of appropriate concentrations of BA.

Furthermore, in general terms, the visibly 2-fold to 3-fold smaller shoots and the development of twisted shoots during the initial stages of development observed in unsliced cotyledon node explants compared to longitudinally sliced cotyledon node explants might be due to inhibition caused by the higher concentration of BA. Similarly, Diallo et al. (2008) noted that increasing kinetin concentrations led to inhibition and resulted in smaller shoots in another cowpea variety, Mougne. Similar results were reported by Chandra et al. (1995) for experiments on *V. radiata* L. where researchers sliced the cotyledon nodes vertically to achieve shoot regeneration.

An increase in BA concentrations resulted in a proportional decrease in shoot length on both explants in the present study, confirming previous findings in cowpea (Diallo et al., 2008; Aasim et al., 2009a, 2010), and narbon vetch (Kendir et al., 2009).

All of the regenerated shoots rooted easily, and the plants were acclimatised in the greenhouse and matured to produce viable seeds. The results are in agreement with those of Aasim et al. (2008a, 2009a, 2009b, 2010) and others (Baba Erdağ et al., 2010; Hasançebi et al., 2011), who also reported the successful rooting and acclimatisation of *in vitro* regenerated cowpea.

## Conclusions

In terms of practical approaches, these findings open a wide field for application in cowpea micropropagation, and the experimental results clearly illustrate the advantage of using longitudinally sliced cotyledon nodes over unsliced cotyledon nodes. This understanding could also have implications for the improvement of this crop through genetic manipulation.

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