Explant source, genotype and plant growth regulators effects on chickpea (Cicer arietinum L.) callus induction

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ABSTRACT: Callus induction was studied in three leaflets and internodes chickpea cultivars, ICC 3996 C, Zouaoui and Flip 82 150 C on MS (Murashige and Skoog, 1962) nutrient medium, supplemented with several hormonal combinations of 2, 4-D (2,4-Dichlorophe noxyacetic acid), NAA (1-Naphthaleneacetic acid) and BAP (6-Benzylaminopurine). The results showed that rates of callus production was significantly influenced at 5 % level, by individual and interactive, genotype, explants source, hormonal balance added to the nutrient medium effects. Among the genotypes tested, Zouaoui cultivar showed better callogenic ability (61, 66%) compared to ICC 3996 C (50, 66%) and Flip 82 150 C (45, 75%). The two types of explants explained different reactivity. Internodes were significantly more callogenic and formed 61, 66 % of produced callus, whereas with leaflets, only 45% were obtained. In addition, from the range of hormonal regime tested, MS medium containing 1 mg/l BAP 0, 5 mg 2, 4-D, or 0,5 mg/l NAA with 2,25 mg/l BAP, expressed the greatest callus formation rate with respectively 76,80% and 71,92%. However, MS containing 2, 4-D or NAA without cytokinine, showed low percentages of produced callus.

Key words: Cicer arietinum L., Genotype, Growth regulators, Internodes, Leaflets.

INTRODUCTION

Chickpea (Cicer arietinum L.) is an important grain legume cultivated on about 10 million ha worldwide with a yield of 8.28 million tons annually (Akidobe and Maredia, 2011). It offered high quality proteins, vitamins and minerals for human consumption especially in developing countries where the nutritional intake from animal sources is limited. It’s also a hopeful crop for alternative agriculture in industrialized countries due to its capacity to fix near 70% of atmospheric nitrogen mainly by its symbiotic relationship with Rhizobium ciceri (Zaman et al., 2010).

Unfortunately and in spite of the large important needs, yield of chickpea has not significantly increased these last decades mostly in Mediterranean basin countries (Benzohra et al., 2010), due to several biotic and abiotic factors. Trends to overcome these production constraints through traditional breeding tools are insufficient and must be assisted by modern biotechnology means. Recent advances in cell and tissue culture allowed the occurrence of new plants with interesting characters (Sutan et al., 2010). Plant tissue culture, especially, callogenesis may induce somaclonal variation or genetic variability source of new useful characters for plant improvement (Shahab-ud-din et al., 2011), but although it seems to be an interesting way, its use requires a prerequisite and reliable standardized protocol. For leguminous plants like chickpea, this is rather difficult because of their recalcitrant nature (Naz et al., 2008, Ochat et al., 2010). Many works on callus initiation in chickpea reported these phenomena (Anwar et al., 2010, Zamane et al., 2010; Zare Mirakabad et al., 2011). Thus, an effective callogenesis protocol, facilitating biotechnological techniques application for transformation and selection of stress resistant plant is needed.

Callogenesis initiation depended on several factors like the nature and concentration of plant growth regulators in the culture medium, the genotype and the explants used (Mathias and Simpson, 1986, Barna and
The aim of this investigation is to study these factors’ effect on *Cicer arietinum* L. callus initiation in order to find a highly efficient protocol for a successful callogenesis.

**MATERIALS AND METHODS**

**Plant material**

The plant materials sources used in this study were three chickpea seeds genotypes (Zouaoui, ICC 3996 C and Flip 82 150 C).

They were sown in 12 x 12 cm pots containing autoclaved soil-peat mixture (3:1, v/v) as 3 seeds by pot repeated 5 times. Plants were watered with tap water daily and grown in a green house at 22°C ± 2.

**Callus induction experiment**

To initiate callogenesis, both leaflets and stem segments of 0, 5 cm length were excised from 03 weeks old plants. They were disinfected by initial dipping in 70° ethanol for 30 seconds followed by a treatment with 5% sodium hypochlorite solution for 1 min, and then explants were rinsed 3 times with sterilized distilled water.

Callus induction was conducted on full strength basic media of Murashige and Skoog (1962), supplemented with 05 plant growth regulator combinations for each genotype and explant (Tab. 1). All the media formulations had 30g/l of sucrose, 15g/l of agar agar and the pH adjusted to 5, 8 before autoclaving at 120 °C for 20mn.

**Table 1. Various hormonal combinations added to MS medium for Cicer arietinum callogenesis induction**

<table>
<thead>
<tr>
<th>Growth regulators Medium</th>
<th>2,4-D mg/l</th>
<th>NAA mg/l</th>
<th>BAP mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS5</td>
<td>-</td>
<td>0,5</td>
<td>-</td>
</tr>
<tr>
<td>MS7</td>
<td>0,5</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>MS11</td>
<td>2</td>
<td>0,5</td>
<td>-</td>
</tr>
<tr>
<td>MS14</td>
<td>-</td>
<td>0,5</td>
<td>2,25</td>
</tr>
</tbody>
</table>

For each explant and individual treatment, 10 fragments were randomly placed in 90 mm Petri dishes, containing culture medium and incubated at a temperature of 25°C± 2 under 16 hours light/ 8 hours darkness photoperiod regime of. The experiment was repeated 5 times. The explants response was followed regularly and the effect of different treatments was quantified after 04 weeks. The percentages were calculated on the basis of the number of callus formed to the whole number of explants tested.

**Statistical analysis**

The callus formation frequency of different explants of chickpea genotypes was analyzed as factorial experiment in a completely randomized design. The data were subjected to analysis of variance by Statistica software, version 10 and means compared by Tukey’s HSD (Honestly Significant Difference) at alpha=0, 05.

**RESULTS**

Explants responses began after a mean of five days, they were illustrated by a swelling up, and after 03 weeks of in vitro culture, an initiation of callogenesis started in the two types of explants for the three genotypes tested. Small Calli occurred all over the surface of the leaflet explants and were predominantly initiated in the cut edges of the stem segments in contact with the culture medium. After 04 weeks, the calli had variable obvious characteristics with different colors and textures depending on the hormonal combination added to the medium. Generally they were large, friable, green to yellow on MS7; dark green with hard surface on MS14 (Fig. 1); moist, small, yellow to beige on MS1 and MS11 and small beige with a hard surface on MS5.
Significant differences were observed in callus initiation response between internodes and leaflets of the three investigated genotypes on all tested media with a percentage ranging from 2 to 100%. The results are summarized in figure 2.

Figure 1: Callus initiation from leaflets and internodes of three *Cicer arietinum* L. genotypes on MS medium supplemented with different plant growth regulators combination after 4 weeks of incubation under a photoperiod regime of 16h light/8h darkness at 25°C ± 2.

a: ICC3996 leaflets on MS7 with 2,4-D/ BAP (0, 5/ 1 mg/l); b: ICC3996 leaflets on MS14 with BAP/NA (2,25/0,5 mg/l); c: Zouaoui internodes on MS14 with EAP/NA (2,25/0,5 mg/l); d: Zouaoui internodes on MS7 with BAP/NA (2,25/0,5 mg/l); e: Zouaoui internodes on MS7 with 2,4-D/ EAP (0, 5/ 1 mg/l); f: F1 Plant 150 C leaflets on MS7 with 2,4-D/ EAP (0, 5/ 1 mg/l).

Significant differences were observed in callus initiation response between internodes and leaflets of the three investigated genotypes on all tested media with a percentage ranging from 2 to 100%. The results are summarized in figure 2.
Figure 2. Callus induction percentages in three chickpea cultivars leaflets and internodes after 04 weeks on MS culture medium supplemented with different hormonal balances.

Analysis of the variance (Tab. 2) showed that the callus initiation in Cicer arrietinum depends on the effect of the hormonal balance, genotype and explants used. It's also influenced by the interaction of these three factors at the same time at 5% level.

Table 2. Variance Analysis of growth regulators formulation effect on callus induction from leaflets and internodes explants of three genotypes of Cicer arrietinum L.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Sum square</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>3922.3</td>
<td>2</td>
<td>1961.1</td>
<td>4.8275</td>
<td>0.010122</td>
</tr>
<tr>
<td>Medium</td>
<td>35755.6</td>
<td>4</td>
<td>8938.9</td>
<td>22.0041</td>
<td>0.000000</td>
</tr>
<tr>
<td>Explant</td>
<td>7656.1</td>
<td>1</td>
<td>7656.1</td>
<td>18.8464</td>
<td>0.000036</td>
</tr>
<tr>
<td>Genotype*Medium</td>
<td>16122.2</td>
<td>8</td>
<td>2015.3</td>
<td>4.9608</td>
<td>0.000039</td>
</tr>
<tr>
<td>Genotype*Explant</td>
<td>14277.8</td>
<td>2</td>
<td>7138.9</td>
<td>15.7532</td>
<td>0.000000</td>
</tr>
<tr>
<td>Medium*Explant</td>
<td>9277.1</td>
<td>4</td>
<td>2319.3</td>
<td>5.7091</td>
<td>0.000374</td>
</tr>
<tr>
<td>Genotype<em>Medium</em>E</td>
<td>18387.3</td>
<td>8</td>
<td>2298.4</td>
<td>5.6578</td>
<td>0.000007</td>
</tr>
<tr>
<td>Error</td>
<td>37780.1</td>
<td>93</td>
<td>406.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant at 5% level.

*Callogenesis vary with different genotypes*

Response in callus initiation is highly genotype dependent. Significant differences in response were observed among the chickpea genotypes investigated. On the bases of means comparison by Tukey’s HSD test (Tab. 3), the Zouaoui cultivar with 61, 66% had the highest rate of formed callus, while ICC 3996 C and Flip 82 150 C cultivars with 50, 66% and 45, 75 % respectively, showed less callus initiation percent. Therefore, the callus induction potential was different between tested genotypes and Zouaoui cultivar produced more callus than ICC 3996 C and Flip 82 150 C.

Table 3. Percentage of callus obtained from leaflets and internodes of three chickpea genotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Leaflets</th>
<th>Internodes</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC 3996 C</td>
<td>44 b</td>
<td>59 b</td>
<td>50.66 a</td>
</tr>
<tr>
<td>Zouaoui</td>
<td>37.89 a</td>
<td>81.30 c</td>
<td>61.66 b</td>
</tr>
<tr>
<td>Flip 82 150 C</td>
<td>50 a b</td>
<td>37.33 a</td>
<td>45.75 a</td>
</tr>
<tr>
<td>Means of explants</td>
<td>45 a</td>
<td>61.66 b</td>
<td></td>
</tr>
</tbody>
</table>

Data are means after 4 weeks of culture from leaflets and internodes of three Cicer arrietinum genotypes. Values followed with same letters are not significantly different indicted by HSD Tukey’s test at P≤ 0.05.
**Callogenesis potential expression depends on the source of explants**

Callus induction is strongly related to source of explants. Means comparison of produced callus percentage of chickpea genotypes related to explants type, showed that callogenic potential expression was higher in internodes compared to leaflets. Hence, 61, 66% was obtained with cut stems and 45 % from leaflets (Tab. 3).

**Callogenesis is highly plant growth combination dependent**

Plant growth regulators had a significant effect on callus induction. The results indicated that all treatments induced calluses. However, according to the mean of formed callus from leaflets and internodes for all genotypes (Tab.4), significant differences were observed.

On the mediums including both an auxin (2, 4-D or NAA) and a cytokinine (BAP), the rate of callogenesis was higher. This was noticed with MS7 containing 0, 5 mg/l of 2, 4-D and 1mg/l of BAP and MS14, including 0, 5 mg/l of NAA and 2, 25 mg/l of BAP where formed callus percentage reached respectively76, 80% and 71, 92%. However, mediums containing only auxins, either MS1 with 2 mg/l of 2, 4-D, MS5 containing 0, 5 mg/l of NAA, or MS11 with 2 mg/l of 2,4-D and 0, 5 mg/l of NAA, showed lowest results with 42,3%, 35,6% and 36,80% respectively. Moreover, these hormonal combinations seem to be disadvantageous to chickpea callus proliferation, since these turned brown probably due to polyphenols accumulation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Percentage of callus (%)</th>
<th>Zouaoui Leaves</th>
<th>Flip82150 Leaves</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1 (2,4-D (2mg/l))</td>
<td></td>
<td>Internodes</td>
<td>Leaflets</td>
<td>42.3 a</td>
</tr>
<tr>
<td>ICC3996C Leaflets</td>
<td>26±9.8 a</td>
<td>58±8 a</td>
<td>76±14.4 a</td>
<td>35.6 a</td>
</tr>
<tr>
<td></td>
<td>100±6 b</td>
<td>50±15.2 bc</td>
<td>86±4 a</td>
<td>56.6±6 a</td>
</tr>
<tr>
<td>MS5 (NAA (0,5mg/l))</td>
<td>2±2 a c</td>
<td>40±0 b c</td>
<td>14±5.8 a</td>
<td>35,6 a</td>
</tr>
<tr>
<td>(2,4-D/BAP(0,5/1mg/l))</td>
<td>100±6 b</td>
<td>50±10 ab</td>
<td>94±4 b</td>
<td>76.80 b</td>
</tr>
<tr>
<td>MS7 (NAA /BAP (0.5/)</td>
<td>2±2 a c</td>
<td>58±4 c</td>
<td>75±18.5 a</td>
<td>36,80 a</td>
</tr>
<tr>
<td>(2,4-D)</td>
<td>32.5±11 c</td>
<td>18±9 a</td>
<td>63±8.8 a</td>
<td>71.92 b</td>
</tr>
<tr>
<td>MS11 (2,4-D+NAA (2/0.5mg/l))</td>
<td>34±6 a c</td>
<td>90±4.1 a</td>
<td>95±5 a</td>
<td>63±8.8 a</td>
</tr>
<tr>
<td>MS14 (NAA+BAP (0.5/2.5</td>
<td>34±6 a c</td>
<td>54±9.9 ab</td>
<td>100±0 b</td>
<td>71.92 b</td>
</tr>
<tr>
<td>mg/l)</td>
<td></td>
<td>95±5 a</td>
<td>63±8.8 a</td>
<td>71.92 b</td>
</tr>
</tbody>
</table>

Data are means ± SD (Standard deviation) after 4 weeks of culture. Means within column followed by the same letter are not significantly different indicated by HSD Tukey’s test at P ≤ 0.05).

**DISCUSSION**

The establishment of an efficient callogenesis protocol, allowing the going beyond chickpea recalcitrant nature to tissue culture and producing high rate callus is an important step for a successful chickpea transformation. From this point of view, the effect of some factors governing the callogenesis is under investigation.

Explants of leaflets and cut stems of three genotypes Cicer arietinum are cultured on MS medium supplemented with different auxins (2,4-D, NAA) and cytokinins (BAP). After one month of in vitro culture, variable callogenesis rate was obtained. This difference is induced by individual and interactive effects of three factors: genotype, source of explants and hormonal balance added to the culture medium. These factors are decisive for chickpea callogenetic potential expression (Arora and Chawla, 2008; Khan et al., 2011).

Callogenesis rate varied significantly with the genotype tested. Among the varieties used, Zouaoui showed the highest reactivity, with 62% of produced callus compared to ICC 3996 C and Flip 82 150 C with respectively 46% and 51%.

Khan et al. (2011) reported difference in the capacity of callogenesis expression for two different indigenous chickpea genotypes, KK1 and Hassan 2K even when subjected to identical in vitro culture conditions. The recalcitrant nature observed in some genotypes may be due to their physiological characteristics (Sané and Mustapha, 2010), specially endogenous hormones levels, or to a genetic inability (Sané et al.,, 2012). Genotype effect on callogenesis is reported as well for chickpea, (Rao and Chopra, 1987; Islam et al.,, 1998; Zare Mirakabad et al., 2010 and Khan et al., 2011), as for other species like tobacco (Ali et al., 2007) and palm tree (Sané et al., 2012).

It was noted that the two explants tested showed different callogenesis capacity. Thus, 62% of internodes produced calluses against 42% with leaflets. Rao and Chopra (1987), Riazuddin (1988), Barna and Wakhlu (1993), and Arora and Chawla (2005) indicated the importance of the explants source on callogenesis response. Explants type, and eventually its anatomy structure, may play a significant role in callus initiation, organs and tissue response according to their reactivity to the culture medium components (Zouzou et al., 2008).
Explants response appeared to strongly depend on plant growth regulators used and their concentrations. Altaf et al. (1999) suggested that different hormones must be used according to the morphogenetic response targeted, the tissue used and its metabolic statute.

The choice of hormones rather than their concentration is determinant to explants callogenesis orientation (Bharathi and Elavarasi, 2012). Auxins and cytokinins, are two well known hormones for their direct and or indirect effects on cells proliferation induction, and on their orientation to a specific organization (Zrÿd, 1988). They may act in synergy or antagonism (Jones and Ljiung, 2011). Auxins, favorable to cellular proliferation (Boxus et al., 1995), are essential for synthesis and play a decisive role in tissue culture (Gautheret, 1959, Neumann et al., 2009). Cytokinins allow cell division and act with auxins to stimulate their multiplication.

Regarding Cicer arietinum, the results showed that the best responses have been recorded on the medium containing 2, 4-D/ BAP or NNA/ BAP, it could be assumed that the combination of auxin and cytokinin is favorable to callogenesis in chickpea. A similar result was reported by Sagare et al. (1993), Huda et al. (2003), Zare Mirakabad et al. (2010), Aasim et al.(2011) and Khan et al. (2011).

Moreover this research showed that either 2, 4-D or NAA when used even separately or in combination, seem to give low produced callus rate for all the tested genotypes. Different results were reported by Poomi et al. (2011); Kumar et al. (2011); Naz et al. (2008) where 2, 4-D alone is regarded as an adequate hormone to promote tissue culture in Cicer arietinum L. According to Khan et al. (2011), even 2, 4-D alone could induce callus production in chickpea, the supplementation of a cytokinin such us BAP is suitable for a better calluses proliferation. Besides, callus browning and viability decreasing occurred when the culture medium was supplemented with only 2, 4-D and/or NAA. This phenomenon probably due to polyphenols accumulation (Zrÿd, 1988), is often reported in chickpea callogenesis (Naz et al., 2008, Zare Mirakabad et al., 2011). These different results prove the necessity to establish a callogenesis protocol for each genotype (Riazuddin et al., 1988 and Altaf et al., 1999).

This investigation conclude that chickpea callogenesis is highly dependent on genotype, explants and hormonal balance, interactive effect. The frequency of produced callus was higher in Zouaoui genotype compared to ICC 3996 C and Flip 82 150 C. The association of 2, 4-D or NAA with BAP allows to obtain a high number of callus, with better expression of cells proliferation ability in the case of internodes explants.

**ACKNOWLEDGMENTS**

The authors are grateful to Dr. M. Labdi (INRA Sidi Bel Abbes, Algeria) for seed material providing and Dr.F.Z. Boukhatem (Oran University) for the manuscript checking.

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