



## Chickpea (*Cicer arietinum* L.) *in vitro* micropropagation

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### Abstract

This research was undertaken to determine chickpea *in vitro* micropropagation optimal conditions from preexisting meristems. Three genotypes; Zouaoui, ILC 483 and INRA 199 mature embryos and nodes were cultivated on Murashige and Skoog (1962) medium without plant growth regulators and with NAA (Naphthyl acetic acid) /BAP (Benzyl amino purine) (0.5/4.5mg/l) and KIN (Kinetin) (0.5, 1mg/l). Explant morphogenetic response was recorded after one month incubation. Results were expressed as direct regeneration frequency (shoot and root development from embryos and axillary buds from nodes). Zouaoui genotype was more callogenic (42.57%) than INRA 199 (32.12%) and ILC 483 (23.43%). Among media, MS without hormones was suitable to induce preexisting meristems development (76.16%), whereas MS with NAA/BAP was favorable to callus formation (70.95%). Indirect buds formation was accomplished on MS added with 0.5mg/l KIN via callusing on mature embryos.

**Keywords:** Chickpea, mature embryos, nodes, *in vitro* culture, callogenesis, buds formation.

### Introduction

Chickpea is an important grain legume cultivated worldwide on more than 12 million ha (FAOSTAT, 2012) and representing an important and available protein, phosphorus, iron and soluble vitamins source. However, its production is limited due to many biotic and abiotic stresses. Conventional plant selection methods remain insufficient and must be assisted by biotechnology tools. This may open new opportunities thought tissue culture. Particular chickpea recalcitrant nature to *in vitro* culture, a reliable micropropagation protocol is prerequisite for further genetic transformation and stress resistant plants selection (Ochatt et al. 2010).

The objective of the present work was to study mature embryos and nodes behavior when cultured *in vitro* to develop and establish a reproducible plant regeneration system in chickpea. This could be extended for genetic transformation.

### Materials and Methods

Seeds of three chickpea genotypes namely, Zouaoui, ILC 483 and INRA 199 were used to provide explants. Mature embryonic axes were excised from imbibed from 24h soaked seeds at 4° C to avoid their germination. Nodal meristems

were cut from 03 weeks old plants cultured on autoclaved peat sand mixture (3:1), (v/v) under green house. The explants were disinfected by immersion in 70% ethanol solution for 30 seconds, then 2 % sodium hypochlorite solution for 01 minute and finally rinsed three times with sterile distilled water. Thereafter, they were cultured on full strength MS medium (Murashige and Skoog, 1962) containing 2% sucrose with pH adjusted at 5.8 and solidified with 7% of agar.

Morphogenetic response of both explant types was studied on MS alone (MS0) and when adding exogenous plant growth regulators M14 (NAA, Naphthyl acetix acid, /BAP Benzyl amino purine, 0.5/4.5mg/l), K1 (KIN kinetin 0.5mg/l) and K2 (KIN 1 mg/l).

Petri dishes containing medium and 8 explants were incubated at 25°C±2 under 16h luminous / 8h darkness photoperiod regime.

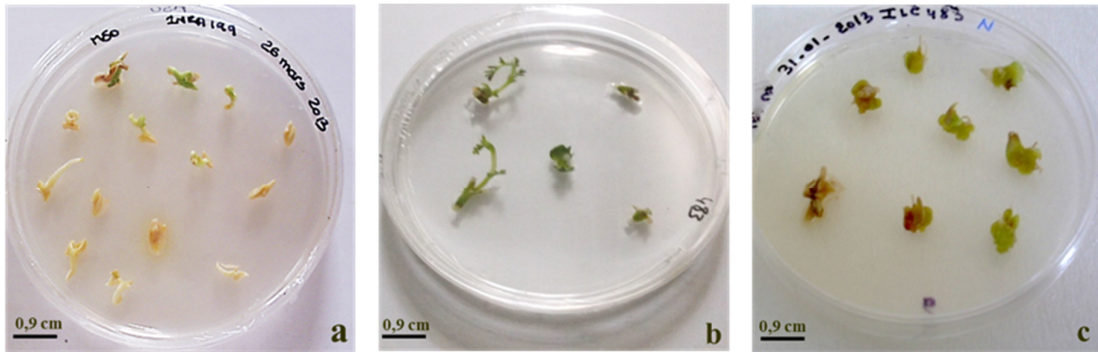
Tests were conducted in completely randomized block (3 genotypes X 02 explants X 03 medium) with 4 replications. After one month morphological response was recorded and results subjected to analysis of variance (ANOVA). Mean differences were compared pairwise using the Duncan multiple comparison test (Statistica version 5, Statistica Software Inc.)

**Results**

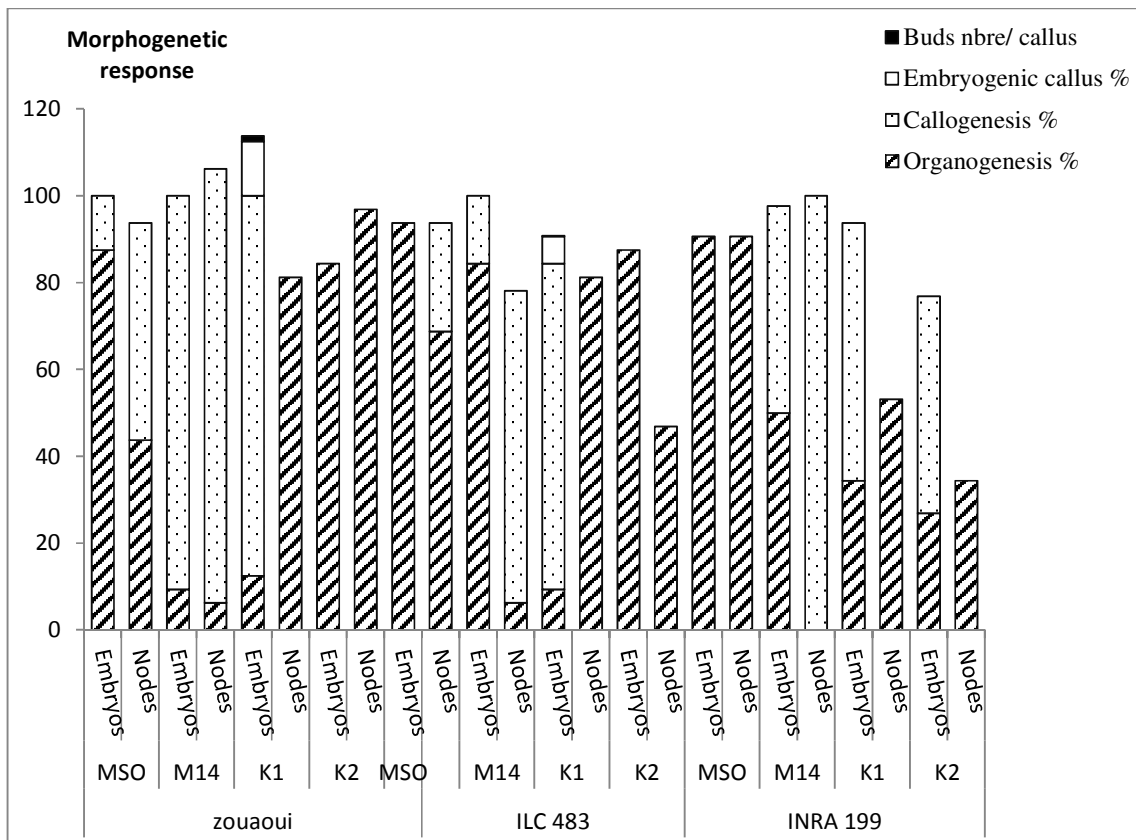
Explants response occurred 05 days (Fig.1a) after culturing. Even mature embryos and nodes swelled and after they were oriented to distinct morphological changes, organogenesis (Fig 1b) and callogenesis (Fig 1c).

Some of embryonic axes showed shoot and root development from the preexisting meristems

and others formed calluses. Nodes explants presented new axillary bud development and in many cases callus formation. After one month data was recorded for mature embryos and nodes morphogenetic response (Fig.2).



**Figure 1.** Morphogenetic response, beginning on embryos (a), organogenesis (b), and callogenesis (c)



**Figure 2.** Effect of different hormonal combinations on morphogenetic response of three Chickpea genotypes mature embryos and node explants

**Table 1.** Different Morphogenetic response means analyses of variance

Effect	ddl	Organogenesis %		Callogenesis %		Embryogenic callus %		Buds number per callus	
		F	P	F	P	F	P	F	P
Genotype (G)	2	2.46	0.0009 *	8.44	0.005*	1.8	0.12	10.5	0.0001*
Medium (M)	3	25.51	0.0000*	55.25	0.000*	5.4	0.002*	18	0.0000*
Explant (E)	1	1.27	0.0027*	3.9	0.0049*	5.4	0.01*	18	0.00065*
GxMxE	6	4.01	0.0001*	3.45	0.0004*	1.8	0.11	10.5	0000*

\*significant at p<0.05

Regarding genotype, means of explants giving organogenesis were not significantly different, whereas in callogenesis, highest rate was obtained with Zouaoui (42.57%) compared to INRA199 (32.12%) and ILC483 (23.43%)( Tab 2).

**Table2.** Chickpea genotype effect *in vitro* morphogenetic response

	Zouaoui	ILC 483	INRA199
Organogenesis (%)	52.73 a	59.76 a	47.50 a
Callogenesis (%)	42.57 a	23.43 b	32.12 b
Embryogenic callus (%)	1.56 a	0.78 a	0 a
Buds number / callus	1.95 a	0.39 b	0 b

Lines followed by same letters are not significantly different at p<0.05

Referring to media, results were significantly different at  $p \leq 0, 05$ . MS0 was favorable to induce highest rate of organogenesis (76.16 %) than K1 (45.3%) and M14 (26.04%). This medium was more suitable to promote callogenesis (Tab.3).

**Table3.** Culture medium effect on chickpea *in vitro* culture morphogenetic response

	MS0	M14	K1	K2
Organogenesis (%)	76.16 a	26.1 b	45.3 c	61.81 d
Callogenesis (%)	14.58 a	71 b	36.9 c	8.33 a
Embryogenic callus	0 a	0 a	3.12 b	0 a
Buds number /callus	0 a	0 a	3.12 b	0 a

Lines followed by same letters are not significantly different at p<0.05

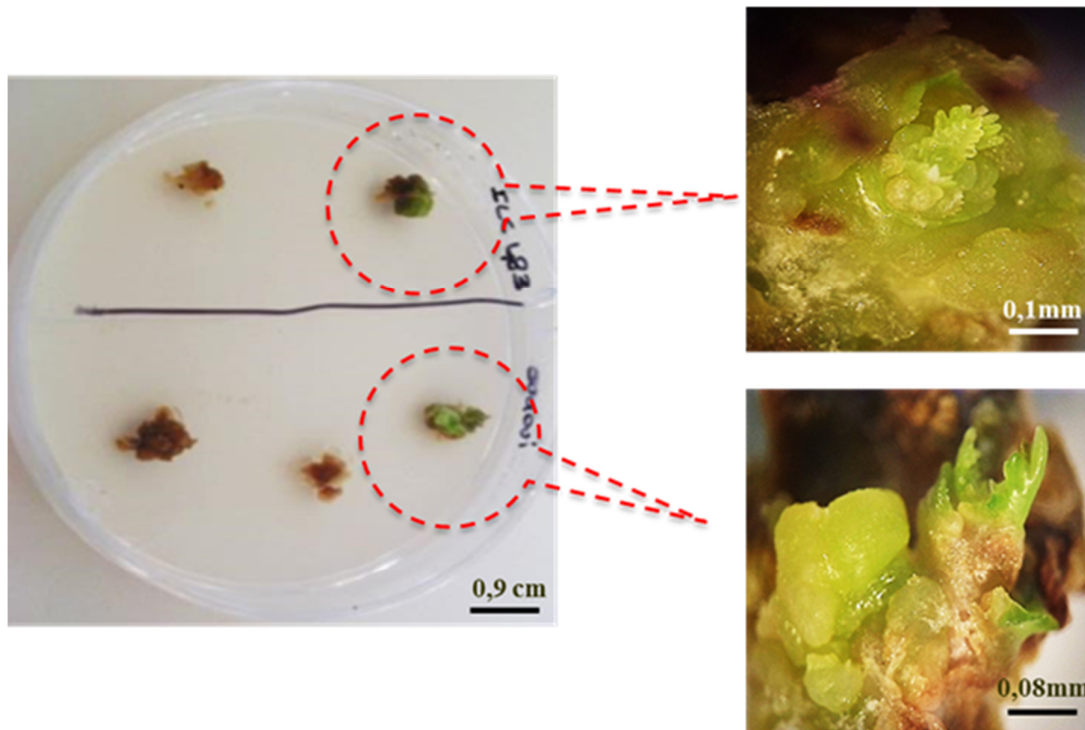
Generally, embryonic axes showed higher capacity of either organogenesis (55.88%) or callogenesis (36.52 %) compared to nodes where 50.78% of explants were able to develop shoots and 28.90% produced calluses (Tab.4).

**Table4** Explant type effect on chickpea *in vitro* culture morphogenetic response

	Mature embryos	Nodes
Organogenesis (%)	55.88 a	50.78 a
Callogenesis (%)	36.52 a	28.90 b
Embryogenic callus (%)	1.56 a	0 b
Buds number / callus	0.39 a	0 b

Lines followed by same letters are not significantly different at p<0.05

Obtained callus varied from green hydrated and friable when cultured on MS0 and M14 to beige nodular and compact on K1. After subculturing on same fresh media, these occasionally showed buds initiation, with apparent leaf primordia. (Fig3) Analyse of variance revealed that this formation is essentially influenced by hormonal combination added to medium. Results obtained on different tested medium were significantly different and MS added with 0.5mg/ l kinetin showed highest capacity to induce indirect embryogenesis from chickpea mature embryos.



**Figure 3.** Buds formation from chickpea callus on MS medium supplemented with 0,5mg/l KIN

### Discussion

This research was undertaken to establish optimal chickpea *in vitro* micropropagation, qualified as recalcitrant by many researchers. (Naz et al., 2008; Anwar et al., 2010; Zamane et al., 2010). Three chickpea genotypes (Zouaoui, INRA 199 and ILC 483) embryos and nodes *in vitro* morphogenetic response was studied on MS culture medium alone or supplemented with exogenous hormones.

Results showed that they are mostly oriented to pre-existing meristems development or to cells dedifferentiation then callogenesis. This is influenced by several factors namely genotype, explant type and culture medium (Yadav et al., 2012). Indeed, under current adopted experimental conditions, results showed these three factors significant effect.

Regarding genotype, Zouaoui, ILC 483 and INRA 199 exhibited relatively NAAlogous organogenesis capacity with respectively 52.73 %, 59.76 % and 47.50 %. Similar results were reported with several cumin genotypes embryos and nodes (Ebraheimi et al. (2007) and in different soybean cultivars (Texiera et al., 2012).

For callogenesis, data indicated significant difference between genotypes response where 42.5 % of Zouaoui explants formed calluses. Khan et al. (2011) indicated that different genotypes

when cultured *in vitro*, expressed variable callogenic capacity even subjected to same experimental conditions. This is observed in chickpea callogenesis (Rao and Chopra, 1987; Islam et al., 1998; Khan et al, 2011), or other species (Ali et al., 2007; Sané et al., 2012). According to Sani and Mustapha (2010), it may be due to genotype physiological characteristics.

*In vitro* cultured explant behavior control is essentially based on exogenous applied hormones nature and concentrations. Altaf et al. (1999) suggested that in tissue culture, hormones choice is oriented according to targeted morphogenetic response, used tissue and its metabolic statute.

Auxins and cytokinins are two well-known hormones used to obtain callogenesis or organogenesis, acting in synergy or antagonism (Zrýd, 1988). MS culture media used alone or added with hormones exhibited different rates either in direct organogenesis and callogenesis for all tested genotype explants. MS without added hormones allowed 76.16% of cultured explants to express their organogenic ability. Kilikova et al. (2004) indicated that seedling *in vitro* development is mainly based on endogenous hormones stock. Otherwise, when adding combined auxin and cytokinin in M14, explants are mostly oriented to callus formation. Comparable findings were motioned for different chickpea explant *in vitro*

culture (Sagare et al., 1993; Huda et al, 2003; Aasim et al. 2011) and other leguminosae (Krishna et al., 2011). Different explants have variable *in vitro* capacity (Nunes et al., 2003). Generally embryonic axes were more reactive than nodes either for direct organogenesis or callogenesis.

Explant type and probably its anatomic structure and reactivity with media components are *in vitro* morphogenetic divergence source (Zouzou et al., 2008).

Furthermore, obtained calluses after subculturing on same fresh media, evolved differently. Only those formed on MS supplemented with 0.5mg/l KIN showed buds formation with apparent leaf primordia. Arora and Chawla (2005) noted that chickpea indirect regeneration need exogenous cytokinin application, used at optimal low concentration (Weerakoon, 2010). MS medium with higher concentration of KIN 1mg/l (K2) was unfavorable for buds formation.

Data showed that this was significantly related explant type and enhanced by culture medium, which is often reported in indirect organogenesis (Ebraheimi et al., 2007). However, it's important to notify that for these experimental conditions, buds formation was genotype independent. This result is especially interesting for genetic transformation where non genetic dependent regenerations are required to avoid phenotypic anomalies and cytogenetic changes (Kumar et al, 2013).

### Conclusion

Chickpea tissue culture morphogenetic response depends on genotype tissue nature and culture medium. Mature embryos or nodes can be oriented to shoot and root development or callogenesis. Buds initiation from callus can be obtained from embryos when cultured on MS supplemented with 0,5 mg/l KIN. However, it would be interesting to test more hormonal combinations to increase their number and improve this protocol to achieve plantlet development and acclimation.

### References

- Aasim M., Day S., Rezaei F., Hajizadeh M., Mahmud, St, Ozcan S. *In vitro* shoot regeneration from preconditioned explants of chickpea (*Cicer arietinum* L.) cv. Gokce. African Journal of Biotechnology., 10(11):2020–2023, 2011.
- Ali G., Hadi F., Tariq M., Ali Khan A. Callus induction and *in vitro* complete plant regeneration of different cultivars of tobacco (*Nicotiana glauca* L.) on media of different hormonal concentrations. Biotechnology. 6(4): 561-566, 2007.
- Altaf N., Iqbal J., Salih Ahmed M. Tissue culture of *microsperma lentis* (*Lens culinaris* Medik) cv. massoor-85. Pak. J. Bot. 31(2):283-292, 1999.
- Anwar F., Sharmila P., Pardha Saradhi P. No more recalcitrant: Chickpea regeneration and genetic transformation. African Journal of Biotechnology. 9(6): 782-797, 2010.
- Arora A, Chawla HS. Organogenic plant regeneration via callus induction in chickpea (*Cicer arietinum* Role of genotypes, growth regulators and explants . Indian Journal of Biotechnology. 4:251-256, 2005.
- FAOSTAT. Food and Agricultural Organization of the United Nations, <http://www.fao.org/faostat>. 2012 consulted august 19th 2014.
- Huda S., Siddique N.A., Khatun N., Rahman M.H., Morshed M. Regeneration of shoot from cotyledon derived callus of chickpea (*Cicer arietinum* L.). Pakistan Journal of Biological Sciences. 6(15):1310-1313, 2003.
- Islam R., Farooqui H., Raizuddin S. *In vitro* genotype phytohormone interaction in chickpea (*Cicer arietinum* L.). Plant Tissue Culture. 8(1): 173-175, 1998.
- Khan S., Ahmad F., Ali F., Khan H., Khan A., SWATI Z. A. Callus induction via different growth regulators from cotyledon explants of indigenous chick pea (*Cicer arietinum* L.) cultivars KK-1 and Hassan-2K. African Journal of Biotechnology. 10(40): 7825-7830, 2011.
- Klíčová S., Šebánek J., Vlašic T. The effect of cytokinins and other plant hormones on the growth of cotyledonary axillars of flax (*Linum usitatissimum*), sunflower (*Helianthus annuus*) and pea (*Pisum sativum*). PLANT SOIL ENVIRON. 50 (4): 182–187, 2004.
- Kumar M., Shukla A.K., Singh H., Verma P.C., Singh P.V. Genotype-independent Agrobacterium mediated transformation of germinated embryo of cotton (*Gossypium hirsutum* L.). International Journal of Bio-Technology and Research. 3(1): 91-90, 2013.
- Murashige T, Skoog F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum. 15:473-497, 1962.
- Naz S, Ali A., Ahmed Siddique F., Iqbal J. Somatic embryogenesis from immature cotyledons and leaf calli of chickpea (*Cicer arietinum* L.). Pakistan Journal of Botany. 48(2), 523-531, 2008.
- Nunes C., F., Dos Santo D., Nazare P. Morphogenesis and regeneration of adventitious shoots in *Jatropha curcas* L.

- Australian Journal of Crop Science. 7 (10): 1511-1519, 2013.
- Ochatt S.J., Atif R.M., Patat- Ochatt E., Jacas M., Conreux C. Competence versus Recalcitrance for *in vitro* Regeneration. World Journal of Agricultural Sciences. 6 (5): 630-634 2010.
- Rao B.G., Chopra V.L.. Genotypic and explants differences in callus initiation and maintenance in chickpea. International Chickpea Newsletter. 17:10-12, 1987.
- Sagare A. P., Suhasini K., Krishnamurthy K.V. Plant regeneration via somatic embryogenesis in chickpea (*Cicer arietinum* L.). Plant Cell Reports. 12:652-655, 1993.
- Sané D., Aberlanc- Bertossi F, Diatta L. I. D., GUÈYE B, DAHER A., SAGNA M., BOGEL A. Influence of growth regulators on callogenesis and somatic embryo development in date palm (*Phoenix dactylifera* L.) sahalian cultivars. The Scientific World Journal. Article ID 837395: 1-8, 2012.
- Sani L., Mustapha Y.. Effect of genotype and 2, 4- d concentration on callogenesis in sugarcane (*Saccharum* spp. hybrids). Bayero Journal of Pure and Applied Sciences. 3(1): 238–240, 2010.
- Texeira L.R., Braccini A.L., Churata B.G.M., Vieira E.S.N., Martins P.K., Schuster I. Evaluation of soybean cultivars on the embryogenic and organogenic potential .Acta Scientiarum. Agronomy Maringá. 33(1): 67-74, 2011.
- Weerakoon S.R. Direct and Indirect Somatic Embryogenesis from Petiole and Leaf Explants of Purple Fan Flower (*Scaevola aemula* R. Br. cv. 'Purple Fanfare'). World Academy of Science, Engineering and Technology. 5:701-709, 2010.
- Yadav S.K., Katikala S., Yellisetty V., Kannepalle A., Naraynaa J.L., Maddi V, Mandapaka M., Shanker A.K., Venkateswarlu Bandi V., Kirti Pulugurtha Bharadwaja K.P.Optimization of Agrobacterium mediated genetic transformation of cotyledonary node explants of *Vigna radiata*. Springerplus. 1(1) 59:1-8, 2012
- Zaman M.A, Manjur Abmk, Ahmed M., Islam M. M.. Effect of 2, 4-D on Callus Induction and Subsequent Morphogenesis in Mature Chickpea (*Cicer arietinum* L.). Embryo Culture, Proc. Sixth Intl. Plant Tissue Cult. & Biotech. Conf., Bangladesh Assoc. Plant Tissue Culture and Biotechnology. 53-58, 2010.
- Zare Mirakabad H., Bagheri A.R., Zare Mehrjerdi M. Efficient protocol for break impasses of regeneration via callus for 20 genotypes of chickpea. International Journal of Plant Production., 4 (2), 115-128, 2010.
- Zouzou M., Kouakou T.H., Koné M., Amani N.G., Kouadio Y.J. Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). Australian Journal of Crop Science. 2(1): 1-9 (2008).
- Zrýd J.P. Cultures de cellules, tissus et organes végétaux. Fondements théoriques et utilisations pratiques. Presses polytechniques romandes (Eds.) 308pp., 1988. Ball, D.M., Collins, M., Lacefield G.D., Martin, NP., Mertens, D.A., Olson, K.E., Putnam, D.H., Undersander, D.J., Wolf, M.W., 2001. Understanding forage quality. American Farm Bureau Federation Publication, 1-01, Park Ridge, IL.