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Research Article

In vitro callogenesis essays to induce somatic embryogenesis of the Tunisian chickpea

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Abstract

The present study was the first report on the somatic embryogenesis of the Tunisian chickpea (*Cicer arietinum* L.) particularly supposed to be recalcitrant and difficult to manipulate *in vitro*. An efficient protocol has been developed for inducing indirect somatic embryogenesis derived from immature zygotic embryo axis, young leaflet and hypocotylar explants. Callogenesis was achieved on full-strength Murashige and Skoog's (1962) (MS) basal medium supplemented with two auxin/cytokinin combinations; 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylamonopurine (KIN) or 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to establish the phytohormones requirement to promote the best induction of friable calli. For somatic embryos induction, embryogenic calli developed from zygotic embryo axes, leaflet and hypocotylar explants were cultured on MS full strength and MS/2, half strength, free of phytohormones media. This study found that all explants exhibited high frequency of callus induction. For immature zygotic embryo axes and young leaflet explants, media containing the combination of 2,4-D and KIN were best effective for inducing callogenesis and friable calli. The most important embryogenic callus percentages were obtained on the culture medium containing the highest concentrations of 2,4-D (2 mg L^{-1}) and KIN (0.5 mg L^{-1}). However, media containing the combination NAA and BAP were best effective for inducing embryogenic calli on hypocotylar explants. A maximal rate of embryogenic calli was reached on medium containing 3 mg L^{-1} NAA and 1 mg L^{-1} BAP. The highest number of somatic embryos was obtained with embryogenic calli derived from embryo axis explants and cultured on MS half strength free phytohormones medium. About 56% of somatic embryos converted successfully into fertile plantlets.

Keywords: callogenesis; chickpea; explants; phytohormones; somatic embryo

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Introduction

Cicer L. genus belongs to the Fabaceae family and the Papilionoideae subfamily (APG IV, 2016). It was the only genus classified in its own tribe Cicereae Alef. comprising 39 *Cicer* species including 31 perennials and eight annuals (Van der Maesen *et al*., 2007). In Tunisia, this genus is represented by only one annual species *C. arietinum* L.; the domesticated chickpea (Le Floc'h *et al.*, 2010). Like other legumes, it contributes to soil improvement through its symbiotic fixation of atmospheric nitrogen (Aouani *et al.*, 2001; Rao *et al.*, 2002). It has also the potential to reduce greenhouse gas emissions (Peoples *et al.*, 2017; Peoples and Williams, 2019). It is considered as the second most important legume grain crop in the world after faba beans (FAO, 2018) growing across a wide range of environments (Sleimi *et al*., 1999; Sleimi *et al*., 2001; Berger and Turner, 2007). It is well known as an important economic species and a valued crop providing nutritious food for an expanding world population (Merga and Haji, 2019). In addition to being considered as a low-cost crop, it is a versatile culture with multiple health benefits, nutritional values and good source of energy (Jukanti *et al.*, 2012; Aasim and Khan, 2019; Fikre *et al.*, 2020). It is rich in proteins, minerals, vitamins, fibers, amino acids, essential fatty-acids, and potentially health-beneficial phytochemicals with high levels of antioxidants; phytosterols, alkaloids, phenols, tannins, flavonoids, glycosides and saponins (Wood and Grusak, 2007; Segev, 2011; Ghribi *et al.*, 2015; Al-Snafi, 2016; Cakir *et al.*, 2019; Madurapperumage *et al.*, 2021). Indeed, it possesses good medicinal value and plays an important role in the prevention and treatment of many chronic diseases (Jukanti *et al.*, 2012). It is also known to have traditional medicinal uses as to treat kidney stones, nephralgia, worms, wart and ringworm (Sezik, 2001; Sargın, 2013), and has an effect on the proliferation of breast and prostate cancer cells (Magee *et al.*, 2012; Kumar *et al.*, 2014).

In Tunisia, chickpea is considered among the mainly grown grain legumes with faba beans, broad beans, peas, lentils and beans (SDBH, 2020). It is grown on an average annual area of 35,000 ha with an average yield of 956 kg ha^{-1} and a total production of 13,520 tonnes (Chebbi *et al.*, 2019). But during the last years, the cultivated areas have significant instability and decrease due to major biotic, particularly, the seed-beetles attack in the field and in storage, and abiotic constraints, principally the extreme temperatures and dry conditions during the flowering and pod formation which affect seriously the productivity (Gurjar *et al.*, 2011; Amri *et al.*, 2014; Jha *et al.*, 2014; Madrid *et al.*, 2015; Pandey *et al.*, 2017; Kaloki *et al.*, 2019). Thus, Tunisia resorted to the importations and the extension of cultivated areas in the north (Amri *et al.*, 2014). These constraints play also a critical role in reducing the inherent potential for genetic improvement of yield traits of chickpea (Singh *et al.*, 2014 a and b).

The usage of biotechnology method especially somatic embryogenesis, an advanced propagation technology, is being implemented for large-scale clonal propagation, independent of the seasons, of elite genotypes of interest (Bhattacharya *et al*., 2010). Somatic embryogenesis has an important place in crop improvement through genetic transformation (Osuji *et al.*, 2016). Then, it can contribute significantly to better sustainability of this important food crop (Sharma *et al.*, 2006). It is also considered as an important plant regeneration pathway from cell cultures used in mass production of plants and synthetic seeds (Philips and Gamborg, 2005). Moreover, according to Aasim *et al.* (2013), *in vitro* regeneration of chickpea is difficult showing minimal success and there is an urgent need to develop a regeneration protocol that can ensure the production of qualitative superior fertile plants. In fact, it is widely regarded, like the other grain legumes, as a highly particularly recalcitrant crop for *in vitro* tissue culture, which requires modern plant regeneration strategies for genetic transformation and selection of improved cultivars (Hussain *et al*., 2000; Ganguly *et al*., 2020).

Despite there is different previously published research works on *in vitro* regeneration and morphogenesis of chickpea (Shri and Davis, 1992; Chandra *et al.*, 1993; Brandt and Hess, 1994; Barna and Wakhulu, 1995; Polisetty *et al.*, 1996, 1997; Paul *et al.*, 2000; Rizvi and Singh, 2000; Huda *et al.*; 2001; Singh

et al., 2002; Jayanand *et al*., 2003; Arora and Chawla, 2005; Chakraborti *et al.*, 2006; Yousefiara *et al*., 2008; Rekha and Thiruvengadam, 2009; ZarMirakabad *et al*., 2010; Banu *et al.*, 2011; Yadav and Singh, 2012; Khamassi *et al*., 2012; Parveen *et al.*, 2012; Ugandhar *et al.*, 2012; Al-Tanbouz, 2013; Aasim *et al.*, 2013; Kadiri *et al.*, 2014; Sunil *et al.*, 2015; Al-Tanbouz and Abu-Qaoud, 2016; Kirtis and Aasim, 2019; 2020; Alghamdi *et al.*, 2020), plant regeneration *via* direct and indirect somatic embryogenesis induction has been previously reported in chickpea mainly on Indian and Pakistan cultivars (Barna and Wakhulu, 1993; Sagare *et al.*, 1993; Kumar *et al.*, 1994; Prakash *et al*., 1994; Suhasini *et al.*, 1994; Kumar *et al.*, 1995; Sudha Vani and Reddy, 1996; Murthy *et al*., 1996; *Ramana et al*., 1996; Sagare *et al*., 1999; Hussain *et al*., 2000; Richa and Singh, 2002; Chauhanand Singh, 2002; Rizvi *et al*., 2002; Guru *et al.,* 2004; Kiran Ghanti *et al*., 2005; Naz*et al*., 2008; Anwar *et al*., 2010; Kiran Ghanti *et al*., 2010; Mishra *et al.*, 2012 and Shukla *et al.*, 2015). In fact, to the best of our knowledge, published works on *in vitro* regeneration of the local Tunisian chickpea, and particularly on its somatic embryogenesis are absent. Previous Tunisian somatic embryogenesis studies has been carried out only on *Cucumis melo* L. (Rhimi *et al.*, 2006), *Vitis vinifera* L. (Bouamama *et al.*, 2007; 2009), *Ceratonia siliqua* L. (Ksia *et al.,* 2008) and *Phoenix dactylifera* L. (Othmani *et al.*, 2018).

Therefore, the present research was conducted to study the effect of different explant types and plant growth regulators combinations in the objective to provide successful and reliable method for the initiation and differentiation of embryos from somatic cells of the local Tunisian *C. arietinum*, which is considered as an essential step in initiating the manipulation of gene transfer to improve different interesting agronomic traits. The defined optimized culture conditions can be useful for biotechnological practices for the genetic improvement of the Tunisian chickpea.

Materials and Methods

Plant material and explants origin

Mature seeds of chickpea 'Amdoun' cultivar of the 'Kabuli' type used in this study are purchased from the local Tunisian market. In laboratory, seeds were visually classified according to their size and their outside aspect. The broken, insect damaged and deformed seeds were discarded. Then, they were soaked for 24 h in tap water, sterilized by soaking for 15 min in a 7% solution of hypochlorite sodium, and washed three times with sterile distilled water. In a laminar flow hood and under sterile conditions, seeds were divided into two groups. For the first group, the seed coat was dissected off and the immature zygotic embryo axes (EA) are removed. They are used as explants. On the other hand, the seeds of the second group, were distributed into *in vitro* culture tubes on Murashige and Skoog (1962) (MS) synthetic medium added with sucrose (30 g L^{-1}), EDTA-Fe (0.1 mM L^{-1}) and 7 g L^{-1} of agar (Bactoagar-Difco). The final pH was set to 5.8 with 1N NaOH or 1N HCl before autoclaving for 20 min, at 121 °C and 103 kPa. After, two other types of explants were excised from 15-day old seedlings raised *in vitro*; fragments of hypocotyls (H) at approximately 0.5 cm in length, and entire young leaflets (L) of 0.5 cm in length and 0.3 cm in width.

Primary culture media for callus induction

Intensity of callus formation and embryogenic potentiality of the three different explants (EA, L and H) cultured *in vitro* were evaluated on fifteen primary culture media prepared from full-strength MS basal medium supplemented with different concentrations of phytohormones in two combinations; NAA and BAP for medium 1 to medium 8 (M1-M8) and 2,4-D and KIN for medium 9 to medium 15 (M9-M15). Detailed composition of defined primary media, numbered M1 to M15 is reported in Table 1.

Table 1. Primary culture media defined by different phytohormones concentrations

Primary media	Phytohormones concentration (mg L^{-1})			
	NAA	BAP	2,4-D	KIN
M1	1	0.5	0	0
M2	1	5	0	0
M3	0.5	1	0	0
M4	1	2	0	0
M5	1	1	0	0
M6	1	3	0	0
M7	0.5	3	0	0
M8	3	1	0	0
M9	0	0	0.25	0.5
M10	0	0	0.25	1
M11	0	0	0.5	1
M12	0	0	0.5	0.25
M13	0	0	1	0.25
M14	0	0	1	0.5
M15	0	0	2	0.5

2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: α-naphthalene acetic acid; BAP: 6-benzylaminopurine; KIN: kinetin

After sterilization, explants were inoculated horizontally on Petri dishes (8×8×2 cm) containing each five explants and 25 ml of medium. The cultures were maintained at a day/night temperature of 22 ± 2/19 ± 2 °C and a light intensity of 6,000 lux provided by cool white fluorescent light with a 16 h photoperiod.

Sub-culture media for somatic embryos induction

The embryogenic calli developed from embryo axis, young leaflet and hypocotylar explants were cultured on MS full strength and MS/2 half strength media, free of plant growth regulators, with sucrose at 50 g L^{-1} in order to induce somatic embryos differentiation on calli. Embryogenic calli were maintained by sub-culturing at three weeks intervals on fresh media.

Data analysis

For explants cultured on the primary culture media, data were recorded after four weeks of culture. The Callus Induction Percentage (CI%), the Embryogenic Callus Percentage (ECP%) and the Organogenic Callus Percentage (OCP%) were calculated as follows:

Callus Induction Percentage (CI%) = No. of explants with callus/ Total No. of explants × 100 (1)

Embryogenic Callus Percentage (ECP%) = No. of embryogenic callus/ Total No. of calli × 100 (2)

Organogenic Callus Percentage (OCP%) = No. of organogenic callus/ Total No. of calli × 100 (3)

After 13 weeks of sub-culturing, the number of somatic embryos developed over the surface of each embryogenic callus clump was counted. Each of the experiments described above consisted of 30 replicates per explant per medium.

Statistical analysis

The data were analysed using analysis of variance (ANOVA) and the significance of the differences between means were determined at $p < 0.05$ using the Duncan's multiple range test (Duncun, 1955). To identify the media concentration in growth hormones which induce the best embryogenic callus percentages according to the type of explants, all the media were subjected to Principal Components analysis (PCA) using the SPSS 10.0 program (Inc. Chicago, IL, USA).

Results and Discussion

Explants reactions on primary culture media

Callus initiation was precocious and occurred one week after culturing the explants. After four weeks of culture on primary culture media, the obtained results of the recorded reactions of the three types of explants were described and reported on Table 2.

Table 2. Callus formation frequency and percentages of embryogenic and organogenic calli recorded in chickpea on embryo axes, leaflets, hypocotylar explants cultivated on primary culture media

Primary culture media	Explant type								
	Embryo Axis			Leaflet			Hypocotyl		
	CI%	ECP%	OCP%	CI%	ECP%	OCP%	CI%	ECP%	OCP%
M1	12.08 ± 0.10 n	1.50 ± 0.58 n	9.90 ± 0.27 g	12.06 ± 0.81 m	1.32 ± 0.50 k	10.09 ± 0.01 h	78.00 ± 0.36 c	53.10±0.26 d	3.50 ± 0.58 f
M2	14.50 ± 0.24 m	0.50 ± 0.02 o	10.80 ± 0.51 f	84.06 ± 0.81 d	3.02 ± 0.02 j	24.06 ± 0.13 d	66.20 ± 0.12 d	48.00±0.36 f	0.60 ± 0.02 j
M3	19.10 ± 0.32 k	8.32 ± 0.50 k	12.50 ± 0.13 e	24.06 ± 0.81 j	–	32.08 ± 0.25 c	100.00 ± 0.01 a	68.06±0.31 b	8.32 ± 0.5 c
M4	22.50 ± 0.34 i	7.50 ± 0.52 l	16.50 ± 0.43 c	34.06 ± 0.81 i	7.05 ± 0.02 h	62.08 ± 0.02 a	100.00 ± 0.22 a	61.00±0.26 c	6.50 ± 0.2 d
M5	37.50 ± 0.56 h	12.50 ± 0.34 h	16.41 ± 0.23 d	22.04 ± 0.63 l	–	12.08 ± 0.01 g	82.00 ± 0.10 b	52.15±0.36 e	2.50 ± 0.34 h
M6	21.60 ± 0.10 j	9.38 ± 1.02 i	19.73 ± 0.51 b	78.08 ± 0.91 f	–	22.04 ±0 .10 f	100.00 ± 0.01 a	35.00±0.35 g	9.38 ± 1.02 a
M7	18.00 ± 0.10 l	3.80 ± 0.32 m	8.33 ± 0.52 i	80.12 ±0.52 e	5.33 ± 0.01 i	51.09 ± 0.02 b	62.90 ± 0.20 e	34.00±0.16 h	3.80 ± 0.32 e
M8	7.50 ± 0.62 o	8.50 ± 0.22 j	20.74 ± 0.31 a	58.06 ±0.57 g	–	22.15 ± 0.01 e	100.00 ± 0.01 a	72.30±0.36 a	8.50 ± 0.22 b
M9	67.50 ± 0.20 g	48.50 ± 0.16 f	5.50 ± 0.18 k	12.00 ±0.36 n	32.50 ± 0.36 g	–	11.50 ± 0.62 h	5.38 ± 1.02 n	–
M10	71.08 ± 0.20 f	53.50 ± 0.39 e	2.30 ± 0.08 l	10.20 ± 0.26 o	45.32 ± 0.61 f	0.42 ± 0.18 j	12.50 ± 0.60 g	10.03 ± 1.00 j	–
M11	72.50 ± 0.28 e	69.40 ± 0.32 d	8.42 ± 0.40 h	23.37 ± 0.20 k	50.12 ± 0.41 c	–	10.50 ± 0.20 i	9.41 ± 0.08 k	–
M12	80.30 ± 0.23 d	42.50 ± 0.06 g	7.10 ± 0.02 j	36.06 ± 0.21 h	48.30 ± 0.31e	1.39 ± 0.04 i	13.80 ± 0.42 f	8.08 ± 1.07 l	3.19 ± 0.08 g
M13	84.50 ± 0.30 c	77.50 ± 0.38 b	1.32 ± 0.45 m	93.30 ± 0.34 b	52.32 ± 0.62 b	–	6.50 ± 0.15 k	5.34± 0.02 o	1.18 ± 0.02 i
M14	89.30 ± 0.21 b	70.00 ± 0.61 c	0.50 ± 0.10 o	87.50 ± 0.22 c	49.78 ± 0.21 d	–	8.50 ± 0.40 j	7.18 ± 0.03 m	–
M15	99.50 ± 0.20 a	82.50 ± 0.25 a	0.98 ± 0.02 n	97.02 ± 0.12 a	58.82 ± 0.67a	–	12.50 ± 0.32 g	10.63 ± 1.45 i	–

CI%: Callus Induction Percentage; ECP%: Embryogenic Callus Percentage; OCP%: Organogenic Callus Percentage
*Notes: Means ± SD with the same letter (s) in the same column are not significantly different (Duncan test, $p < 0.05$) ($n = 30$)

Immature zygotic embryo axes reactions on primary culture media

For embryo axis explants cultured on the fifteen primary media tested, the M9 to M15 media showed the most important stimulation of callus initiation which percentages ranged from 67.50 ± 0.20% (M9) to 99.50 ± 0.20% (M15). These media contain MS basal medium supplemented with the combination of 2,4-D (auxin) and KIN (cytokinin) exogenous hormones at different concentrations. Balancing level auxins and cytokinins in the basal medium is very important because it governs the dedifferentiation or differentiation mechanisms of explant (Bourgaud *et al*., 2001). Méndez-Hernández *et al.* (2019) highlighted the interactions between the different plant growth regulators, mainly auxins, cytokinins, ethylene and abscisic acid, during the induction of somatic embryogenesis and emphasized the importance of signaling response on plant somatic embryogenesis. In addition, it is important to note that the intensity of callogenesis was proportional with the increasing of 2,4-D concentrations in the culture media, from 0.25 to 2 mg L^{-1}. In fact, the callus initiation from

embryo axes achieved 84.50 ± 0.30 and 89.30 ± 0.21% on M13 and M14 containing 1 mg L^{-1} 2,4-D in presence of 0.25 and 0.5 mg L^{-1} KIN, respectively. It appeared that the combination of 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KIN was most effective for inducing callogenesis from embryo axis explants reaching the highest percentage (99.50 ± 0.20%) (Table 2). Actually, 2,4-D is known as an auxin applied to induce callus growth because it can revert explant cells to a dedifferentiated state and to begin to divide. Moreover, kinetin was added to stimulate cell division and control morphogenesis of the cells (George *et al.*, 2008). Generally, cytokinin was important for callus and initiation of somatic embryogenesis. It can modify the physiological state of the initial explants and initiate a somatic embryogenesis program (Veltcheva *et al.*, 2005). However, the other eight treatments with NAA and BAP balance did not give good signs of callus appearing. The highest percentage of callus induction, 37.50 ± 0.56%, was obtained on M5 medium containing a ratio NAA/BAP equal to 1.

Calli varied significantly in type, form and texture, ranging from embryogenic calli that appeared friable, nodular and pale yellow, and organogenic ones that are compact, hard and green. Some others tended to become necrotic and turned black. The use of 2,4-D combined to KIN produced friable calli. The most important embryogenic callus percentages are obtained on culture media containing the highest concentrations of 2,4-D (1 and 2 mg L^{-1}) and KIN (0.5 and 0.25 mg L^{-1}). In fact, embryogenic callus rate reached 69.40 ± 0.32, 70.00 ± 0.61, 77.50 ± 0.38 and 82.50 ± 0.25% on M11, M14, M13 and M15, respectively, corresponding to the Group 2 of media defined by the Principal Component Analysis (PCA) (Figure 1). According to Ratjens *et al.* (2018), embryogenic callus has a defined and known genotype compared to seedlings, besides its high regeneration potential and stability of the regenerated plants. Conversely, the combination of these two exogenous hormones (in M9 to M15) did not stimulate the formation of organogenic calli. However, the combination of NAA and BAP was most effective for inducing organogenic calli from embryo axes explants (19.73 ± 0.51% on M6 and 20.74 ± 0.31 on M8).

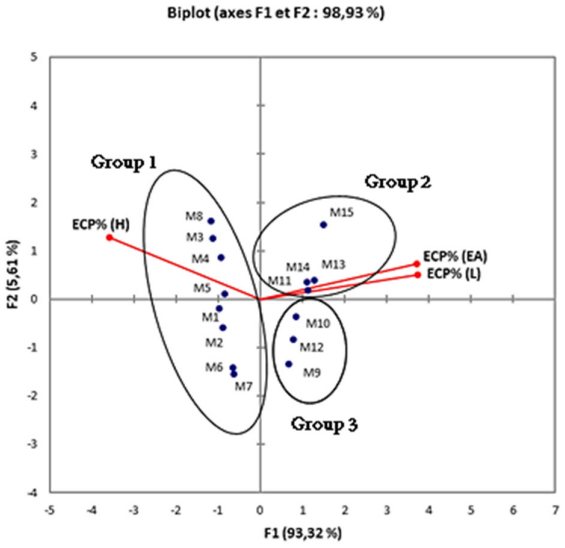

Figure 1. Distribution of primary culture media (1-15) in the plans defined by the axis F1 and axis F2 of the Principal Component Analysis (PCA) based on the percentage of embryogenic callus induced on embryo axes (ECP/EA%), leaflets (ECP/L%) and hypocotylar (ECP/H%) explants

Earlier published works of Sagar *et al.* (1993; 1999) and Suhasini *et al.* (1994) have been developed an efficient protocol for inducing direct somatic embryogenesis from chickpea embryo axes. In both works, somatic embryogenesis was induced on MS medium with 3 mg L^{-1} of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Suhasini *et al.* (1994) recorded 65% of explants forming directly globular embryos which were transferred to half strength MS medium containing 0.1 mg L^{-1} abscisic acid for maturation and subsequently sub-cultured

on half strength MS medium containing zeatin (1 mg L^{-1}). For Sagar *et al.* (1993), 70.6% of embryogenic induction from immature embryo axes has been recorded. More recently, Mishra *et al.* (2012) induced embryogenic callus formation from mature embryonic axes on MS medium supplemented with 5 mg L^{-1} 2,4-D then sub-cultured on the same medium with reduced auxin concentration to 0.05 mg L^{-1}. Shukla *et al.* (2015) was successfully induced somatic embryogenesis from embryonic axis-derived calli of chickpea. On explants cultured in MS medium containing 3 mg L^{-1} 2,4-D, callus induction was optimal (82%) with a considerable conversion of calli to embryogenetic pathway. These results are quite close to ours (99.50%) recorded on MS medium (M15) supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KIN.

Reaction of the leaflet explants on the primary culture media

Callus initiation and growth from young leaflet explants was first observed three or four weeks after culturing. A considerable callus induction was occurring primarily at the veins, and then covered the entire leaflet section. In the media containing 1 or 2 mg L^{-1} 2,4-D combined with 0.25 or 0.5 mg L^{-1} KIN, the explants showed maximal rates of callus formation (87.50 ± 0.22 to 97.02 ± 0.12%) (M13-M15) (Table 2). At lower concentrations of 2,4-D (0.25 and 0.5 mg L^{-1}), poor callus induction was observed (10.20 ± 0.26; M10). Concerning the synergic effect of NAA and BAP, the most efficient callus induction percentages (84.06 ± 0.81, 80.12 ± 0.52 and 78.08 ± 0.91%) (M2, M6, M7) are observed in media supplemented with 5 or 3 mg L^{-1} BAP in combination with 0.5 or 1 mg L^{-1} NAA. If BAP was added at lower concentrations (0.5 or 1 mg L^{-1}), less intense callus induction was obtained (M1; 12.06 ± 0.81%, M5; 22.04 ± 0.63% and M3; 24.06 ± 0.81%).

Friable and white embryogenic calli were formed on most medium formulations containing 0.25-2 mg L^{-1} 2,4-D with all combinations tested of KIN; M9, M10 and M12 forming the Group 3, and M11, M13-M15 forming the Group 2 (Figure 1). In fact, there was a strong interaction between 2,4-D and KIN in induction of embryogenic calli on leaflet explants. Optimal frequencies 58.82 ± 0.67 and 52.32 ± 0.62% were recorded in MS medium supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KIN and 1 mg L^{-1} 2,4-D and 0.25 mg L^{-1} KIN, on M15 and M13 media (Group 2), respectively. In contrary, the organogenic calli initiation was absent (M9, M11, M13, M14 and M15) or very low (0.42 ± 0.18%; M10, 1.39 ± 0.04%, M12). These findings are in agreement with the earlier report of Kumar *et al.* (1994). Foliar explants of the local Indian chickpea showed embryo induction in all the media containing 2,4-D and KIN combinations with varied responses. Embryogenic calli were more obtained (90%) on media with a high ratio of 2,4-D relative to kinetin, particularly with 1.25 mg L^{-1} 2,4-D and 0.25 mg L^{-1} kinetin in the dark corresponding to the best responsive induction medium. The same protocol was repeated for three more cultivars (Kumar *et al*., 1995). Barna and Wakhulu (1993) obtained also somatic embryos from leaflet callus of chickpea. The highest mean number of calli showing embryogenesis was obtained on MS medium containing 25 µM 2,4-D. Rao (1990, 1991) reported that 0.5 mg L^{-1} each of 2,4-D and BAP gave the best frequency of embryogenic leaflet-derived callus. In addition, 2,4-D (2 mg L^{-1}) supplemented to B5 basal medium (Gamborg *et al.*, 1968) was found to be optimum for production of yellow, smooth and nodular embryogenic calli from cotyledon explants of chickpea (Sudha Vani and Reddy, 1996). Immature cotyledons gave rise directly to somatic embryos when cultured on B5 medium supplemented with 7.8 µM 2,4,5-T and 4.4 µM BAP (Ramana *et al.*, 1996). Singh and Chand (2003) obtained a maximum percentage for embryogenic callus formation (89%) on MS medium supplemented with 9.04 mol L^{-1} 2,4-D and 0.46 mol L^{-1} KIN from semi-mature cotyledon explants of *Dalbergia sissoo* Roxb., a leguminous tree. A more recent research work of Chauhan and Singh (2002) revealed that the combination of 1.25 mg L^{-1} 2,4-D and 0.25 mg L^{-1} KIN in MS medium proved to be very effective in inducing a direct somatic embryogenesis from chickpea leaf explants. Naz *et al.* (2008) demonstrated that auxins/cytokinins combinations promoted induction of indirect embryogenesis from young leaf and immature cotyledon explants of chickpea. The best response was obtained in MS medium supplemented with 3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP. They confirmed that the leaf explant was fairly good source for the embryo induction through callus. However, Kiran Ghanti *et al.* (2010) revealed that NAA was better for direct somatic embryo induction

from immature cotyledons compared to other auxins; 2,4-D and 2,4,5-T. A concentration of 6.0 mg L^{-1} NAA produced the highest frequency (100%) and maximum number (40.2) of somatic embryos.

On the other hand, in our study, the most important rate of organogenic callus formation on leaflet explants (M_4; 62.08 ± 0.02%) was induced by the NAA and BAP combinations (1 mg L^{-1} and 2 mg L^{-1}, respectively). The obtained calli on media containing various concentrations of NAA and BAP were green in colour with compact texture, moderate growth rate and promote indirect rhizogenesis.

Reaction of hypocotyl explants on primary culture media

Among the fifteen primary media tested, media containing NAA and BAP combination (M1, M2, M3, M4, M5, M6, M7 and M8) were best effective for inducing callogenesis. In fact, on these media, the hypocotylar explants swelled after two weeks of culture and showed varying and important intensity of cell proliferation. On M3, M4, M6 and M8 media 100% of the explants are callogenic. However, hypocotylar explants cultivated on media supplemented with 2,4-D and KIN did not enhance an important callogenesis induction. Percentages did not exceed 13.80 ± 0.42% (M12) (Table 2). Indeed, the initiation of embryogenic calli was also favored by the NAA/BAP combination on M1-M8 media reaching a maximum rate of 72.30 ± 0.36% (M8 medium). These media constituted the Group 1 defined by PCA (Figure 1). The obtained calli are parenchymatous, friable in texture and white-yellowish in color. In addition, the hormone combinations tested were not effective on the initiation of organogenic callus on hypocotylar explants (Table 2).

In literature, chickpea regeneration occurred under the influence of different types and concentrations of growth regulators (2,4-D, IBA, NAA, BAP, KIN etc.), and it was direct or indirect with an intervening callus using most often initial explants as embryo axes, young leaflets and cotyledonary segments. Somatic embryogenesis was rarely occurred on hypocotyl and only by direct regeneration. Our results are in agreement with those obtained by Murthy *et al.* (1996) who induced profuse nodular calli on the hypocotyledonary notch of the chickpea seedlings cultured on MS medium supplemented with a combination of NAA and BAP growth regulators (5 µM of each). Another earlier study of Sudha Vani and Reddy (1996) confirm our result. In fact, maximal callus initiation (86.6, 96 and 100%) from hypocotylar explants of different cultivars of the Indian *C. arietinum* was obtained on media containing B5 basal medium supplemented with 1 mg L^{-1} BAP and 0.4 mg L^{-1} NAA. Our results confirmed also those found by Hussain *et al.* (2000) who revealed that the hypocotyl tissue showed somatic embryogenesis which regenerated shoots in the presence of a NAA and BAP combination, in order to carry out genetic transformations in chickpea plants. However, Kiran Ghanti *et al.* (2005) induced directly from hypocotyl explants a best somatic embryogenesis on MS medium fortified with different types of auxins (2,4-D, 2,4,5-T, NAA, picloram and dicamba) singly or in combination with kinetin. They demonstrated that picloram was better for somatic embryo induction and its concentration influenced the frequency of somatic embryogenesis in chickpea.

Notable differences were recorded regarding the induction of embryogenic calli on the different explants tested. In fact, explant selection was very crucial to promote callus induction because explant response is highly genotype dependent and not all the types of explant are suitable to induce embryogenic callus (Trigiano and Gray, 2005). In our study, the best reactions were obtained with embryo axes, followed by hypocotyls and then by leaflets. Higher percentages of embryogenic callus initiation (exceeding 70%) have been recorded for embryo axes (82.50 ± 0.25%; M15, 77.50 ± 0.38%; M13, 70.00 ± 0.61%; M14 and 69.40 ± 0.32% M11; Group 2) and for hypocotyls (72.30 ± 0.36%; M8; Group 1) against a maximum rate of 58.82 ± 0.67% (M15; Group 2) with leaflet explants. Those selected media and explants can be used to optimize hormonal combinations for increased embryogenic calli initiation in Tunisian chickpea.

Somatic embryos induction on sub-culture media

When transferred to MS and MS/2 regulator-free media enriched with 50 g L^{-1} of sucrose, the white-yellowish and translucent embryogenic calli continued to proliferate. In fact, osmotic potential provided by sucrose is important in enhancement of somatic embryogenesis (Nhut *et al.*, 2012). On week-10 of incubation, early somatic embryos gave rise on embryonic axes (Figures 2A and 2B), leaflets (Figures 2C-H) and hypocotyls-derived calli surface (Figures 2I-K). They appeared mostly as distinct white globular pro-embryoids, and others were at the early heart-shaped stage (cordiform embryos). On week-13, complete somatic embryos were initiated which had attained the torpedo and cotyledonary stages.

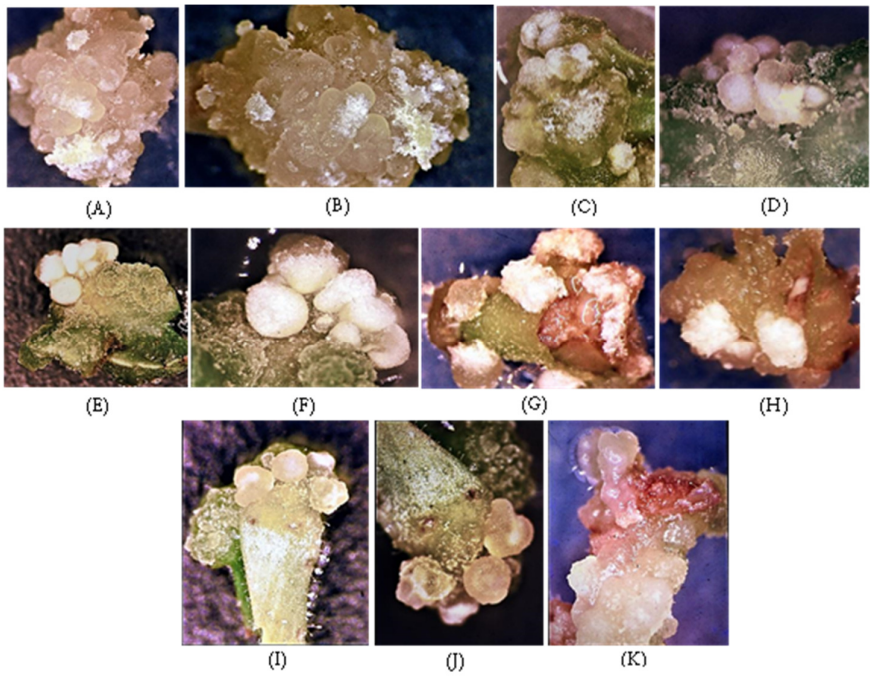

Figure 2. Differentiation stages of chickpea somatic embryos on embryogenic callus after sub-culturing. (A) and (B) Different development stages of initiated somatic embryos on embryonic axes calli; (C)-(H) Globular white somatic embryos observed at the surface of the foliar embryogenic calli; (I)-(K) Globular, cordiform and torpedo somatic embryos formed on hypocotylar calli

Statistical analysis ($p < 0.05$) showed that there was a significant difference in the number of somatic embryos produced by embryogenic calli initiated on embryo axes, leaflets and hypocotylar explants (Figure 3). The explant-derived calli, that produced the highest mean number of somatic embryos, were immature zygotic embryo axes, followed by young leaflets, then by hypocotyls, on MS (16.31 ± 1.03 against 8.65 ± 0.98 and 4.32 ± 1.07, respectively) and on MS/2 media (20.51 ± 1.01 against 10.65 ± 0.85 and 7.96 ± 1.52, respectively).

The mean numbers of somatic embryo on MS/2 are higher than those obtained on MS medium (10.65 ± 0.85 against 8.65 ± 0.98 and 7.96 ± 1.52 against 4.32 ± 1.07). This finding agrees with the earlier report of Sagare *et al.* (1993) who obtained somatic embryos from immature cotyledonary explants and embryo axes derived callus of Indian *C. arietinum* on MS medium supplemented with 3 mg L^{-1} (2,4,5-T). They gave raise to complete plantlets on subsequent transfer to half strength MS medium supplemented with zeatin (1 mg L^{-1}). In addition, our results confirmed those of Singh and Chand (2003), who obtained an average number of 26.5 of somatic embryos per callus clump derived from semi-mature cotyledon explants of *Dalbergia sissoo* Roxbon after 15 weeks of culture on MS/2 medium. Also, the hormone free media (MS and Gamborg) enhanced the development of somatic embryos of *Vicia faba* L. through different stages (Bahgat *et al.*, 2009). Rizvi *et al.*

(2002) obtained a maximum number of chickpea somatic embryos (17) from callus cultured in MS medium supplemented with 10 µM TDZ and 5 mM proline.

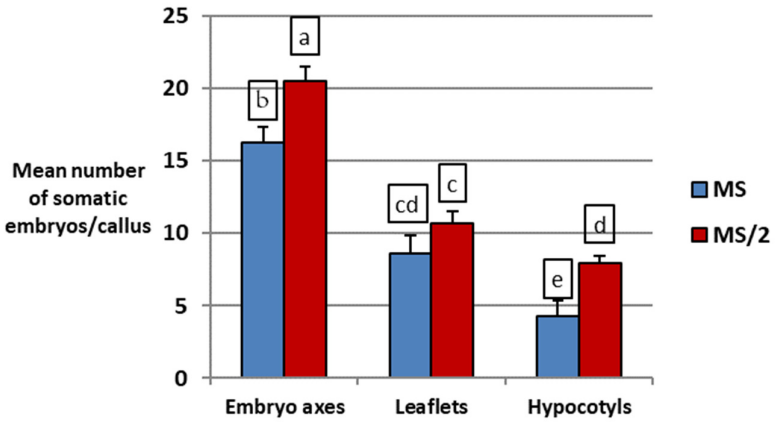

Figure 3. Mean number of somatic embryos per callus derived from embryogenic axe, young leaflet and hypocotyl explants and cultured in MS and MS/2 sub-culture media

The mean numbers of somatic embryo on MS/2 are higher than those obtained on MS medium (10.65 ± 0.85 against 8.65 ± 0.98 and 7.96 ± 1.52 against 4.32 ± 1.07). This finding agrees with the earlier report of Sagare *et al*. (1993) who obtained somatic embryos from immature cotyledonary explants and embryo axes derived callus of Indian *C. arietinum* on MS medium supplemented with 3 mg L^{-1} (2,4,5-T). They gave raise to complete plantlets on subsequent transfer to half strength MS medium supplemented with zeatin (1 mg L^{-1}). In addition, our results confirmed those of Singh and Chand (2003), who obtained an average number of 26.5 of somatic embryos per callus clump derived from semi-mature cotyledon explants of *Dalbergia sissoo* Roxbon after 15 weeks of culture on MS/2 medium. Also, the hormone free media (MS and Gamborg) enhanced the development of somatic embryos of *Vicia faba* L. through different stages (Bahgat *et al.*, 2009). Rizvi *et al.* (2002) obtained a maximum number of chickpea somatic embryos (17) from callus cultured in MS medium supplemented with 10 µM TDZ and 5 mM proline.

Maturation of somatic embryos and germination to plantlets

For maturation and stimulation of their germination into plantlets, obtained somatic embryos were transferred individually into *in vitro* tubes on MS medium without phytohormones. About 56% of somatic embryos converted into plantlets after 25 days of culture (Figures 4A-D), are transferred on Jiffy-pots, containing peat commercial soil substrate (Figure 4E), and were placed in a climatic growth chamber (24 °C, 60% relative humidity, 8 h obscurity/16 h light).

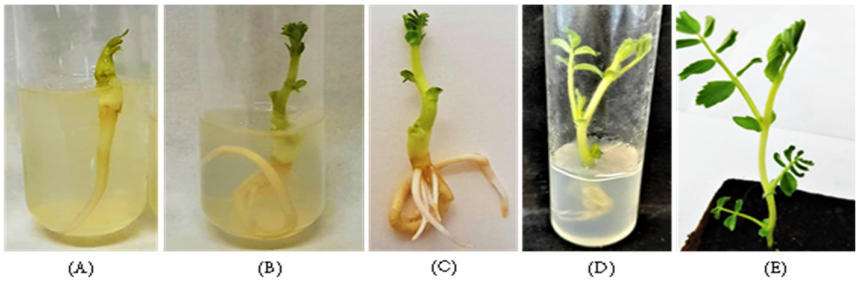

Figure 4. Plant regeneration *via* somatic embryogenesis of the Tunisian chickpea. Fully developed somatic embryo in plantlet with well-emission of root system and first leaves (A, B, C and D). Somatic embryo regenerated plant with elongation of the shoot-root axis in soil (E)

Transferring germinating embryos to hormone free MS basal medium helped in the development of complete plantlets with appreciable frequencies. Our results confirmed the high rates of survival chickpea plantlets obtained by Shukla *et al.* (2015) and Naz *et al.* (2008) on this same medium (83.3 and 89.14%, respectively). However, according to Chauhan and Singh (2002), MS/2 medium can enhance, from 50 to 75%, the conversion of somatic embryos into plantlets.

Conclusions

Our present study emphasized the induction of indirect somatic embryogenesis of the Tunisian chickpea. The obtained results allowed us to select of potential explant, and appropriate concentration and combinations of auxins and cytokinins promoting the best embryogenic calli initiation rates and somatic embryo development. This reported efficient regeneration protocol can be effectively used as a useful tool for genetic modification by inserting novel genes in chickpea plants for further improvement of this important crop in Tunisia.

Authors' Contributions

SK provided the plant material, performed the experiments and wrote the draft of the manuscript; BB monitored the cultures and regularly record the results; HB statistically analyzed the data; SSE supervised the experiments, wrote and discussed results and FHS revised the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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