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ORIGINAL ARTICLE

High efficiency plant regeneration and genetic fidelity of regenerants by SCoT and ISSR markers in chickpea (*Cicer arietinum* L.)

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Abstract

High efficient and repeatable in vitro regeneration protocol was established from embryo axis, half-seed, axillary meristem, and cotyledonary node explants of chickpea. Various concentrations and combinations of various plant growth regulators (PGRs) were employed to induce multiple shoots, shoot elongation and rooting of shoots to obtain complete plantlets of chickpea. The pretreatment of seeds with 6-benzyl aminopurine (BAP) at 1.0 mg l^{-1} was found to significantly increase the multiple shoot regeneration from the all explants tested. Among three PGRs such as BAP, kinetin (KIN) and thidiazuron (TDZ) tested for multiple shoot induction; BAP at 2.0 mg l^{-1} produced the maximum number of shoots in all tested explants. The maximum number of shoots (48.80 shoots/explant) was attained from the embryo axis explant followed by half-seed (32.76 shoots/explant), axillary meristem (28.34 shoots/explant) and cotyledonary node explant (18.47 shoots/explant) on medium augmented with 2.0 mg l^{-1} BAP along with 0.05 mg l^{-1} Indole-3-butyric acid (IBA). The optimum percentage of shoot elongation response was recorded (96.68%) on medium fortified with IAA (0.05 mg l^{-1}), GA_3 (1.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) with an average shoot length of 8.82 cm. The elongated shoots were successfully rooted in medium augmented with 2.0 mg l^{-1} IBA. The complete plants were acclimatized in the greenhouse with a survival rate of 72%. The plantlets regenerated from four explants appeared to be morphologically similar to mother plants. The genetic fidelity of in vitro regenerated plants was evaluated using Start Codon Targeted and Inter simple sequence repeats molecular markers. The in vitro regenerated plants from all four explants were found to be the true to type with their mother plant. The in vitro protocol presented in the study should offer as a feasible system for chickpea genetic transformation.

Key message

An efficient and reproducible in vitro regeneration protocol was established for chickpea. Application of different concentrations and combinations of PGRs was found to enhancemultiple shoot induction, shoot elongation, rooting and acclimatization of in vitro regeneratedplants in field conditions, and further evaluated genetic fidelity using molecular markers.

Keywords Axillary meristem · Chickpea · Cotyledonary node · Embryo axis · Half-seed · Genetic fidelity · Molecular marker · Shoot regeneration

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Abbreviations

PGRs	Plant growth regulators
SCoT	Start codon targeted polymorphism
ISSR	Inter simple sequence repeats
NAA	1-Naphthalene acetic acid
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
BAP	6-Benzyl amino purine
KIN	Kinetin
TDZ	Thidiazuron
GA_3	Gibbelleric acid

Introduction

Grain legumes can supply a sustainable approach to food and nutritional security for the increasing population of the globe. Innovative breeding techniques have been applied to strengthen genomic resources, improve the yield and nutritional value of legume crops, together with enhanced resilience to climatic change (Considine et al. 2017). Legume seeds are the most important nutritional source, as they contain proteins and essential amino acids vital for fulfilling the nutritional need of humans and animals mainly in developing countries like India (Jacob et al. 2016). Chickpea is cultivated in more than 50 countries and traded in 140 countries covering all continents of the globe (Jain et al. 2013; Gaur et al. 2014). India, Australia, Myanmar, Pakistan, and Turkey are amongst the most important contributors to chickpea production. In 2016, Global production of chickpea is reported to be more than 12 million metric tons (FAO 2018). India is the leading producer and accounts for 65% of world production of chickpea by producing about 7.8 million tons annually (FAO 2018). Chickpea production has been constrained by several biotic and abiotic stresses (Croser et al. 2003). There is a high demand to produce chickpea genotypes with superior seed quality, seed quantity, and stress-tolerance to feed the increasing worldwide population. Classical breeding methods have been useful to improve legumes. However, this has resulted in limited success for the transfer of desired traits in chickpea due to its lower genetic variability among the germplasm accessions (Varshney et al. 2009; Gatti et al. 2016). The application of gene transfer techniques has made some progress in the genetic improvement of chickpea (Das and Parida 2014; Leonetti et al. 2018). However, success is plagued by reproducible and dependable plant regeneration protocols and hence there is a greater need to develop such protocols across diverse genotypes and tissues for use in gene transfer aimed at chickpea improvement (Leonetti et al. 2018; Das Bhowmik et al. 2019).

Despite several successful plant regeneration and genetic transformation protocols reported from diverse explants of chickpea (Atif et al. 2013; Leonetti et al. 2018), efficiencies are very low due to the recalcitrant nature for in vitro propagation from various explants of chickpea (Yadav et al. 2017). Furthermore, an efficient plant regeneration technique is required to the transfer of agronomically useful traits into chickpea genotypes (Batra et al. 2002; Sanyal et al. 2003, 2005; Bhattacharjee et al. 2010; Tripathi et al. 2013; Das Bhowmik et al. 2019). Explants with a massive number of totipotent cells will be helpful for improved regeneration and simple strategy under in vitro circumstances. Several reports follow a regeneration method that starts with explant selection and the following exposure to a most favourable plant growth regulator combination and concentration to induce developmental pathways (Zhihui et al. 2009). Determination of the best possible PGRs concentrations like auxins and cytokinins are extremely vital for proper plant regeneration from diverse explants (Daffala et al. 2011). Plant regeneration through direct shoot induction has been reported in chickpea from various explants viz. immature or mature embryo axes with or without cotyledon, decapitated embryo axis, half-seed with cotyledon, seed, cotyledonary node, axillary meristem, hypocotyls, epicotyls, and shoot apex (Uncuoglu et al. 2008; Anwar et al. 2010; Das and Parida 2014).

Genetic variations may be induced in plantlets regenerated from tissue culture, and hence, it is necessary to evaluate the genetic homogeneity of micropropagated plants. In recent years, several DNA based molecular markers are used to assess the genetic stability of regenerated plants. Among several molecular markers, start codon targeted (SCoT) and inter simple sequence repeats (ISSR) markers are extensively applied to identify the genetic homogeneity of tissue cultured plants (Singh et al. 2013; Rohela et al. 2019; Saeed et al. 2019). It is worthwhile to utilize more than one marker to screen the genetic homogeneity of tissue culture-derived plants because every molecular marker has its advantages and disadvantages in detecting the genetic stability (Vasudevan et al. 2017). ISSR is an easy, efficient, and generally used technique to determine the genetic stability of micropropagated plants (Rohela et al. 2018; Saeed et al. 2019). SCoT markers are highly polymorphic and reproducible have many advantages over other markers (Cabo et al. 2014). These markers were effectively used to detect the genetic homogeneity of several in vitro propagated plants (Sathish et al. 2018; Ajithan et al. 2019; Elayaraja et al. 2019).

The present investigation has been undertaken to find out the comparative significance of explant and PGRs for plant regeneration from decapitated embryo axis (EA), half embryo with cotyledon (Half-seed-HS), axillary meristem (AM) and cotyledonary node (CN) explants of chickpea cultivar JAKI9218 using various concentration of three plant growth regulators such as BAP, KIN, and TDZ alone, various concentrations of BAP in combination with three auxins for multiple shoot bud induction, and to determine the role of auxins on rooting for the successful recovery of complete plantlets. Moreover, an attempt has been made to assess genetic fidelity in regenerated plants derived from different explants using ISSR and SCoT molecular markers. Such a morphogenetic system would form an initial material for further studies on genetic transformation in chickpea.

Materials and methods

Seed material and preparation of explants

Chickpea seeds (genotype JAKI9218) were kindly provided by Germplasm Resource Unit, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, Telangana State, India. The healthy and uniform seeds were washed under running tap water with 2–3 drops of Tween20 for 20 min, then sterilized with 70% ethanol for 1 min and followed by a 10 min treatment with 0.1% $HgCl_2$, then thoroughly washed with sterile distilled water for five times and soaked overnight in sterile distilled water. Seed coat was removed and germinated on MSB5 (Murashige and Skoog 1962; Gamborg et al. 1968) medium fortified with 3% sucrose and diverse concentrations of BAP (1.0–3.0 mg l^{-1}). Embryo axes (EA) and half-embryo with cotyledon (HS-Half seed) explants were prepared after germination for 1–2 days. The seeds were placed on medium and incubated for 2 days in the dark at 24 ± 2 °C and later shifted to 16/8 h light/dark photoperiod with a light intensity of 50 µEm^{-2} s^{-1} with cool white fluorescent lights. Axillary meristem and cotyledonary node explants prepared from 5 to 7 day-old aseptic seedlings were used for further experiments.

Embryo axis (EA)

The sterile overnight soaked seeds were blotted dry then used for preparation of an embryo axis (EA) explants. The seed coat of the soaked seeds was removed. Embryonic axis explants were obtained by removing cotyledons, shoot (plumule) and root (radicle) apices. These decapitated embryo axis explants were subjected to multiple shoot bud initiation (Pathak and Hamzah 2008).

Half embryo with cotyledon (half-seed-HS)

The sterilized seeds were soaked in a flask containing (150 ml Erlenmeyer) 30 ml of sterile distilled water (30 seeds per flask) for 16–18 h. These imbibed seeds were allowed to germinate under dark conditions for 2 days at 24 ± 2 °C. The seeds were transferred to the sterile petriplate and dissected after removing the seed coat. A longitudinal cut was made throughout the plumule to divide the cotyledons. The plumule and the edge of radicle were removed to obtain the half embryo with cotyledon (half-seed-HS) explants. The dissected half embryos with single cotyledon (half-seed) were used as explants (Chakraborti et al. 2006).

Axillary meristem explants (AME)

The sterilized seeds were de-coated and germinated on medium augmented with BAP (1.0 mg l^{-1}). Axillary meristem explants were obtained from 5 to 7 day-old seedlings grown under 16 h photoperiod and maintained at 24 ± 2 °C. The axillary meristem explants were prepared with the bifurcation of seedling and then removed the apical meristem, axillary buds and root tip. Two explants were prepared from each seedling (Srivastava et al. 2012).

Cotyledonary node (CN) explants

The sterilized seeds were placed on medium amended with BAP (1.0 mg l^{-1}) for germination. About 30 ml of the culture medium was dispensed into 150 ml flasks and ten seeds were inoculated per flask then incubated for 2 days under total darkness at 24 ± 2 °C for 3–5 days under 16/8 h light and dark conditions at 24 ± 2 °C in the culture room. The 5–7 day-old seedlings were chosen to prepare cotyledonary node explants by dissecting out both cotyledons, epicotyl, and hypocotyl (Yadav and Singh 2012).

Culture media and conditions

For all experiments, the basal culture media containing MS salts and B5 vitamins was used for all experiments conducted in the present study. All media were fortified with 3% sucrose and solidified with 0.8% (w/v) agar before autoclaving. The pH of the media was adjusted to 5.6–5.8 using 0.1 N NaOH/0.1 N HCl before autoclaving at 121 °C (103.4 pKa) for 15–20 min. All cultures were incubated at 24 ± 2 °C with a 16/8 h photoperiod under fluorescent light (50 µEm^2 s^{-1}).

Effect of PGRs on multiple shoot induction, shoot elongation, rooting and acclimatization

Decapitated embryo axis, half-embryo with cotyledon (half-seed), cotyledonary node and axillary meristem explants were transferred into shoot induction medium (SIM) [20 ml/tube] in the culture tubes containing sucrose (3%) along with various concentrations of PGRs namely; KIN, BAP (1.0–5.0 mg l^{-1}), and TDZ (0.5–2.5 mg l^{-1}) as an individual component to compare their effect on the regeneration ability (Table 1). TDZ was filter-sterilized and then added to the warm autoclaved medium. Half-seed explants were inoculated in such a way that radicle was embedded in the medium whereas cotyledonary node explants placed vertically with the shoot apical region facing up on the medium. The embryo axis, half seed with cotyledon, axillary meristem, and cotyledonary node explants were cultured on the shoot induction medium (SIM) augmented with diverse

Table 1 Effect of different plant growth regulators (BAP, KIN, TDZ) on shoot bud induction from different explants of chickpea cv. JAKI 9218

Plant growth regulators (in mgl^{-1})	Mean number of shoots/explant			
	EA	HS	AME	CN
BAP				
0.0	02.94 ± 0.76^{ef}	02.26 ± 0.84^{ef}	01.82 ± 0.76^{ef}	02.26 ± 0.56^{ef}
1.0	03.62 ± 0.38^{e}	03.48 ± 0.46^{de}	02.52 ± 0.32^{de}	02.46 ± 0.63^{de}
2.0	11.34 ± 0.56^{ab}	10.62 ± 0.38^{a}	08.28 ± 0.44^{a}	06.38 ± 0.74^{a}
3.0	10.76 ± 0.42^{ab}	07.28 ± 0.65^{bc}	06.64 ± 0.56^{bc}	04.62 ± 0.54^{bc}
4.0	08.84 ± 0.46^{c}	06.62 ± 0.58^{bc}	05.68 ± 0.52^{bc}	04.24 ± 0.62^{bc}
5.0	06.48 ± 0.62^{d}	04.28 ± 0.65^{de}	03.86 ± 0.66^{de}	02.62 ± 0.42^{de}
KIN				
1.0	02.28 ± 0.66^{e}	03.24 ± 0.54^{de}	02.28 ± 0.82^{de}	02.06 ± 0.38^{de}
2.0	07.46 ± 0.62^{a}	06.32 ± 0.50^{a}	05.68 ± 0.42^{ab}	04.57 ± 0.82^{ab}
3.0	05.26 ± 0.48^{b}	04.68 ± 0.54^{bcd}	04.64 ± 0.38^{ab}	03.62 ± 0.34^{ab}
4.0	04.64 ± 0.60^{cd}	04.22 ± 0.38^{bcd}	03.26 ± 0.60^{bc}	03.14 ± 0.28^{bc}
5.0	04.46 ± 0.32^{cd}	04.08 ± 0.65^{bcd}	02.82 ± 0.46^{cd}	02.32 ± 0.36^{cd}
TDZ				
0.5	03.64 ± 0.48^{e}	03.18 ± 0.60^{e}	02.20 ± 0.65^{e}	02.06 ± 0.30^{e}
1.0	10.24 ± 0.60^{a}	08.25 ± 0.26^{ab}	07.18 ± 0.42^{a}	05.38 ± 0.23^{ab}
1.5	08.38 ± 0.62^{bc}	07.82 ± 0.60^{ab}	05.42 ± 0.62^{bc}	04.22 ± 0.54^{ab}
2.0	07.04 ± 0.46^{bc}	06.22 ± 0.28^{bc}	04.38 ± 0.20^{bc}	04.18 ± 0.48^{bc}
2.5	06.28 ± 0.86^{cd}	05.68 ± 0.46^{cd}	04.06 ± 0.56^{cd}	03.46 ± 0.44^{cd}

Each experiment was repeated thrice with 30 replications. Values represent the mean ± standard error. Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level

EA embryo axis, *HS* half embryo with cotyledon (Half seed), *AME* axillary meristem explant, *CN* cotyledonary node

concentrations of BAP (1.0, 2.0 and 3.0 mg l^{-1}) and in combination of IAA/IBA/NAA (0.05 and 0.5 mg l^{-1}) as individual component to compare their effect on multiple shoot induction. After fifteen days of initial culture, the four explants with shoot buds were subcultured onto a fresh medium for two times successively with a 15 days interval.

After 6 weeks, the multiple shoot buds obtained from four different types of explants were shifted to shoot elongation medium (SEM) augmented with different combinations and concentrations of BAP (1.0 mg l^{-1}), KIN (1.0 mg l^{-1}), IAA (0.05 mg l^{-1}), NAA (0.05 mg l^{-1}) and Gibberellic acid (GA_3) [1.0 mg l^{-1}] to determine their role on shoot elongation. After 15 days of culture on SEM, a subculture was done for all the types of explants with fresh SEM containing the same plant growth regulator concentrations and kept for another 15 days.

The elongated shoots were separated from a bunch of multiple and used to optimize their rooting using different auxins (IAA, IBA, and NAA). Individual elongated shoots (~ 2–3 cm) from all four explants viz., decapitated embryo axis, half-seed, axillary meristem, and cotyledonary node were isolated from a bunch of shoots/shoot buds of chickpea genotype JAKI9218. The separated shoots, a total of 120 shoots per each auxin treatment (30 shoots from each explant type) were inoculated onto the root induction medium (RIM) supplemented with diverse concentrations of NAA, IAA, and IBA (1.0 and 2.0 mg l^{-1}) to optimize the ideal concentration of auxin for root induction. The rooting percentage was determined in different treatments after 15 days.

The complete plantlets were gently separated from the vessel and washed under running tap water to eliminate traces of gelling agent sticking with the root surface, and then shifted to paper cups (8 × 7 cm) containing sterile sand, vermiculite, and soil in equal ratio. The plantlets were dipped in an aqueous solution of 0.1% bavistin (a systemic fungicide-BASF India, Ltd, Mumbai) for 5–10 min. Plantlets were kept in test tubes with ~ 10 ml of sterile water for 2–3 days. All plantlets were enclosed with small polyethylene bags with a minute prick and placed in a growth chamber maintained at 24 ± 2 °C with relative humidity (RH) with 85% for 2–3 weeks. Once in 2 days, water was poured to the plantlets. After the formation of new leaflets, the plantlets were shifted to pots containing a mixture of sterile soil, vermiculite, and sand (in equal ratio) and maintained under the greenhouse conditions.

Plant DNA isolation and genetic homogeneity assessment with SCoT and ISSR markers

For genetic fidelity assessment, twelve in vitro propagated field transferred plantlets (after 6 weeks) (three from each explant regenerated) were selected randomly along with the mother plant. The 500 mg of leaf sample from each plant was used for the extraction of genomic DNA by CTAB method (Doyle and Doyle 1987). Genetic stability assessment was carried out with ten sets of each SCoT and ISSR primers (Tables 4 and 5). The PCR reactions were performed using a total amount of 25 µl having a 2X PCR master mix (GCC Biotech, India), 10 pmol of primer and 50 ng/µl of plant DNA sample. PCR amplification reaction was performed in a thermal cycler (Bio-Rad, USA) with an initial denaturation of DNA at 94 °C for 5 min, followed by 30-s denaturation at 94 °C, and 45-s annealing at 52 °C for SCoT, 50 °C for ISSR and 2 min extension at 72 °C, the final extension of 10 min at 72 °C and a holding temperature of 4 °C. The amplified products were separated in 0.8% agarose gel using 1X TBE (Tris Borate EDTA) buffer. The sizes of amplicons were calculated using a 1 kb ladder (Thermo Scientific, USA). The gel images were photographed using the gel doc system (Bio-Rad, USA). The scorable and clear PCR amplified bands were only measured.

Statistical analysis

Data were scored after each treatment, like multiple shoot induction from all four types explants consisting of 30 explants for each treatment. Data on multiple shoot initiation shoot elongation and rooting were analyzed using a completely randomized block design. All experiments were repeated thrice. The data was analyzed and presented in tables and figures as means ± standard error of three independent experiments. The mean separations were carried out using Duncan's new multiple range tests (DNMRT) and significance was determined at a 5% level ($p < 00.5$) using SPSS 16.0 (SPSS Inc. USA).

Results and discussion

Explants

The present investigation was carried out to establish plant regeneration protocol through multiple shoot induction from four diverse types of explants of chickpea genotype JAKI9218. A necessary prerequisite for genetic modification of chickpea cultivars is the efficient regeneration and the multiple shoot bud formation system that could provide the means for improving chickpea cultivars using either *Agrobacterium* or particle bombardment method. In the present study, decapitated embryo axes, half-seed (2-day-old), axillary meristem and cotyledonary node (7-day-old) explants were assessed for multiple shoot production in chickpea using different combinations and concentrations of PGRs. For the successful establishment plant regeneration in different genotypes, various combination and concentration of PGRs and explants play a vital role in chickpea (Sanyal et al. 2005; Chakraborti et al. 2006; Bhattacharjee et al. 2010; Srivastava et al. 2012; Tripathi et al. 2013). The exogenous addition of PGRs changes the growth of axillary meristems and promotes the meristematic cell proliferation in the axillary buds by mounting the number of shoot bud primordial that originate from the pre-existing axillary meristems (Srivastava et al. 2012; Kumari et al. 2018). Hence, the present study was conducted to optimize plant regeneration procedure with suitable combinations and concentrations of PGRs and explant type in chickpea.

Pretreatment of seeds

The seeds were germinated on medium fortified with different concentrations of BAP (1.0–3.0 mg l^{-1}) within 2 days. It was noted that the increased BAP concentration declined the germination response and provoke differences in seedling morphology. The MS medium augmented with BAP at 1.0 mg l^{-1} was found to be optimum for seed germination and the seedling possesses distended cotyledons, thick and tiny hypocotyl with the enlarged end of the roots devoid of lateral roots and visible axillary buds. The half-seed, axillary meristem, and cotyledonary node explants were prepared from these seedlings showed the greatest percentage of regeneration response and generated the maximum number of shoots when compared to explants obtained from without seed pretreatment (Fig. 1). The embryo axes explants appear to be suitable explant for direct shoot regeneration in several chickpea cultivars (Pathak and Hamzah 2008; Srivastava et al. 2012; Kumari et al. 2018). Other advantages of embryo axes, half-seed, axillary meristem, and cotyledonary node explants are the constant availability of explants source (in vitro seedling) and less percentage of culture contamination. Cytokinin pre-treatment while raising the aseptic seedlings showed differences in regeneration response when cultured on MSB5 medium fortified with diverse plant growth regulator concentrations for multiple shoot bud formation from various explants. In the present study, explants from seedlings grown in medium amended with BAP (1.0 mg l^{-1}) influences the regeneration response. Our observations are in agreement with the earlier reports in soybean that preculturing seeds in cytokinin medium make manipulation of explants during dissection easier because it causes enlargement of the cotyledon as well as allowing initial development of many numbers of shoots (Hada et al. 2018). The encouraging effect of pretreatment of seeds with cytokinins

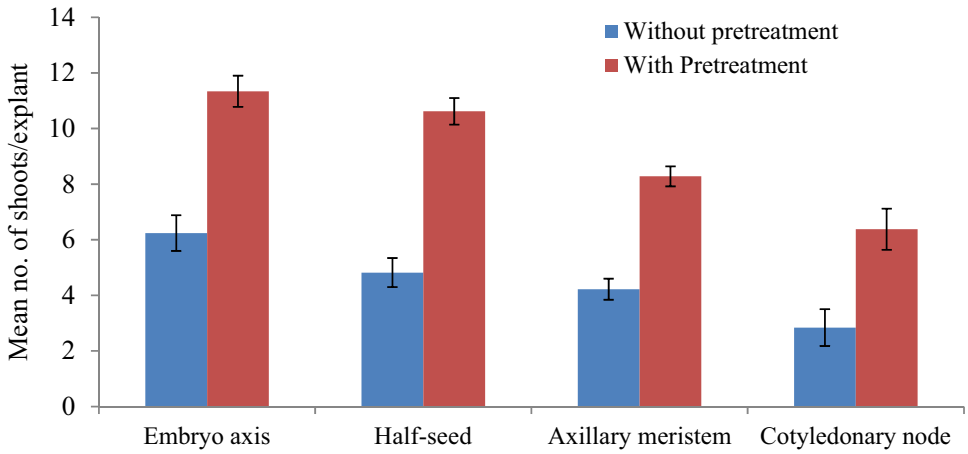

Fig. 1 Effect of pretreatment of multiple shoot induction from four different explants of chickpea cv. JAKI9218 on MSM5 medium supplemented with BAP (1.0 mg l^{-1})

has already been observed in several legumes such as *Cajanus cajan* (Sharma et al. 2006), *Glycine max* (Hada et al. 2018), *Vigna radiata* (Amutha et al. 2006), *Vigna unguiculata* (Raveendar et al. 2009) including *Cicer arietinum* (Sanyal et al. 2005; Srivastava et al. 2012). In general, the pretreatment of seeds enhances the number of shoots when compared with the explants isolated from untreated seedlings (Raveendar et al. 2009; Hada et al. 2018).

Effect of plant growth regulators on multiple shoot induction

To evaluate the morphogenic ability via multiple shoot induction, four different explants viz., decapitated embryo axes, a half embryo with one cotyledon, cotyledonary node, and axillary meristem explants were placed on medium supplemented with various concentrations of BAP, KIN, and TDZ. Among four explants tested, embryonic axis explants were superior for multiple shoot induction (11.34 shoots/explant) than other explants tested. The multiple shoot induction percentage and time of induction was varied depending upon the type of explant and PGR concentrations used. While embryo axes, half seed, and cotyledonary explants exhibited induction of multiple shoot buds within 7 days of culture, axillary meristem explants induced multiple shoot buds in 12 days. Among the different concentrations of BAP, KIN and TDZ tested, BAP at 2.0 mg l^{-1} was most efficient for shoot bud induction in all four explants of chickpea (Table 1). The embryo axes explants cultured on BAP at 2.0 mg l^{-1} were found to be efficient with the production of 11.34 shoots followed by half-seed explants (10.62 shoots/explant), axillary meristem explants (8.28 shoots/explant) and cotyledonary node (6.38 shoots/explant) (Table 1). An increased or decreased concentration of BAP induced lower response of multiple shoot induction in all the explant cultures (Table 1). An increase in the concentration of other cytokinins KIN and TDZ also showed a similar turn down in the number of shoots in the explants (Table 1). The results confirmed that among the plant growth regulators, BAP was superior cytokinin for multiple shoot induction from all four types of explants than KIN and TDZ. Successful regeneration protocols were reported from various explants of several genotypes of chickpea on medium fortified with BAP (Polisetty et al. 1997; Chakraborti et al. 2006; Ghanti et al. 2009; Srivastava et al. 2012; Tripathi et al. 2013; Sunil et al. 2015; Amer et al. 2019).

The multiple shoot bud induction was affected by a wide variety of factors viz, explant, concentration, and combinations of PGRs. The various concentrations of BAP (1.0, 2.0 and 3.0 mg l^{-1}) combined with various concentrations of IAA (0.05 and 0.50 mg l^{-1}), IBA (0.05 and 0.50 mg l^{-1}) and NAA (0.05 and 0.50 mg l^{-1}) to determine their synergistic role in multiple shoot induction from four explants of chickpea. Among different concentrations of auxins in combination with BAP (1.0, 2.0 and 3.0 mg l^{-1}) tested for multiple shoot induction, BAP at 2.0 mg l^{-1} with low auxin (0.05 mg l^{-1}) was most effective in multiple shoot induction and produced a maximum number of shoots in all explants (Tables 2 and 3). Among the four explants tested, embryo axes were found to be best concerning the number of shoots per explant on all culture media irrespective of explant type. Among the various combinations of BAP and IBA tested, BAP (2.0 mg l^{-1}) with IBA (0.05 mg l^{-1}) was showed the maximum percentage of response with augmented production of multiple shoots in all types of explants. The embryo axis explants produced 48.80 shoots (Fig. 2a), 32.71 shoots in half-seed explants (Fig. 2b), 28.36 shoots in the axillary meristem (Fig. 2c) and 22.47 shoots in cotyledonary node explants (Fig. 2d) (Tables 2 and 3). The four explants viz. embryo axes, half-seed, axillary meristem, and cotyledonary nodes explants were evaluated for multiple shoot induction and regeneration on six combinations and concentrations of NAA (0.05 and 0.5 mg l^{-1}) and BAP (1.0, 2.0 and 3.0 mg l^{-1}). Among diverse combinations and concentration

Table 2 Effect of BAP in combination with IAA or IBA or NAA on multiple shoot induction from decapitated embryo axis (EA) and half-seed (HS) explants of chickpea cultivar JAKI 9218

Plant growth regulators (in mgl^{-1})		EA		HS	
		Percentage of response (%)	Mean no. of shoots/explant	Percentage of response (%)	Mean no. of shoots/explant
BAP	IAA				
0.0	0.00	46.42 ± 0.76^{g}	02.94 ± 0.76^{g}	34.82 ± 0.56^{g}	02.26 ± 0.84^{g}
1.0	0.05	68.62 ± 0.26^{bc}	18.52 ± 0.36^{bc}	64.82 ± 0.56^{e}	12.64 ± 0.82^{ef}
2.0	0.05	76.35 ± 0.36^{ab}	22.64 ± 0.58^{a}	74.56 ± 0.63^{a}	18.82 ± 0.48^{a}
3.0	0.05	66.46 ± 0.42^{bc}	16.22 ± 0.62^{bcd}	67.34 ± 0.54^{cd}	14.56 ± 0.36^{cd}
1.0	0.50	64.34 ± 0.48^{bcd}	12.66 ± 0.48^{ef}	68.74 ± 0.48^{cd}	15.34 ± 0.62^{bc}
2.0	0.50	72.45 ± 0.36^{ab}	19.38 ± 0.54^{bc}	72.45 ± 0.64^{b}	16.48 ± 0.58^{bc}
3.0	0.50	62.64 ± 0.76^{de}	11.42 ± 0.62^{ef}	60.36 ± 0.46^{f}	11.66 ± 0.42^{ef}
BAP	IBA				
1.0	0.05	78.52 ± 0.64^{bc}	27.62 ± 0.46^{bc}	77.64 ± 0.82^{bc}	22.82 ± 0.56^{cd}
2.0	0.05	88.46 ± 0.76^{a}	48.80 ± 0.38^{a}	86.48 ± 0.56^{a}	32.76 ± 0.36^{ab}
3.0	0.05	74.82 ± 0.54^{de}	26.34 ± 0.52^{bc}	73.62 ± 0.24^{cd}	20.52 ± 0.48^{cd}
1.0	0.50	68.44 ± 0.36^{de}	23.72 ± 0.64^{de}	71.58 ± 0.36^{de}	18.63 ± 0.42^{ef}
2.0	0.50	76.48 ± 0.62^{bcd}	28.46 ± 0.48^{b}	75.88 ± 0.62^{bc}	31.48 ± 0.62^{ab}
3.0	0.50	63.48 ± 0.84^{ef}	18.62 ± 0.63^{f}	65.72 ± 0.84^{f}	16.22 ± 0.46^{ef}
BAP	NAA				
1.0	0.05	72.42 ± 0.48^{cd}	24.45 ± 0.68^{e}	74.66 ± 0.54^{cd}	16.64 ± 0.68^{bcd}
2.0	0.05	82.36 ± 0.56^{a}	28.62 ± 0.44^{a}	82.48 ± 0.38^{a}	26.48 ± 0.66^{a}
3.0	0.05	68.82 ± 0.54^{de}	22.48 ± 0.56^{de}	78.64 ± 0.74^{bc}	18.82 ± 0.43^{bcd}
1.0	0.50	66.62 ± 0.48^{ef}	21.26 ± 0.82^{de}	$71,12 \pm 0.46^{ef}$	14.46 ± 0.48^{ef}
2.0	0.50	76.48 ± 0.62^{b}	26.63 ± 0.76^{b}	76.48 ± 0.32^{bc}	16.52 ± 0.52^{bcd}
3.0	0.50	62.58 ± 0.52^{ef}	16.34 ± 0.32^{f}	68.43 ± 0.52^{ef}	12.39 ± 0.62^{ef}

Each experiment was repeated thrice with 30 replications. Values represent the mean ± standard error. Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level

of BAP and NAA tested, NAA (0.05 mg l^{-1}) and BAP (2.0 mg l^{-1}) combination were found to be the best combination concerning the percentage of response (86.62%) and number shoots per explant (16.62–28.62). The maximum number of shoots was produced from embryo axes (28.62), half-seed explants (22.48), axillary meristem (20.85) and cotyledonary node (16.62) (Tables 2 and 3). Media fortified with BAP and IAA enhanced the number of shoots per explant in all four explant types. BAP (2.0 mg l^{-1}) and IAA (0.05 mg l^{-1}) medium was found to be highly efficient combination concerning to percentage of response and number shoots per explant. Maximum number shoots were produced from embryo axes with 22.64 shoots followed by half-seed with 18.82 shoots, axillary meristem with 20.86 shoots and cotyledonary node with 14.26 shoots in chickpea (Tables 2 and 3).

In the present investigation, the exogenous application of BAP in combination with auxins (IAA/IBA/NAA) in SIM resulted in the maximum shoot induction frequency in all four explants. Auxins are known to promote multiple shoot induction in various legumes like pigeonpea (Geetha et al. 1998), soybean (Hada et al. 2018), vigna (Tang et al. 2012) including chickpea (Srivastava et al. 2012). Among diverse combinations and concentrations of BAP and IBA tested, BAP (2.0 mg l^{-1}) and IBA (0.05 mg l^{-1}) combination best for the percentage of response and number shoots per explant in all explants (Tables 2 and 3). BAP alone induced multiple shoots whereas a low concentration of auxin used with BAP showed a synergistic effect on induction of the maximum number of shoots in chickpea (Batra et al. 2002; Chakraborti et al. 2006; Ghanti et al. 2009; Srivastava et al. 2012). In the present investigation, by manipulating the combinations and concentrations of BAP, IAA, IBA, and NAA, in the culture medium, it has been demonstrated that chickpea explants could produce robust and normal plantlets. Multiple shoots were induced from various explants of several chickpea genotypes on media fortified with BAP and IBA (Batra et al. 2002; Ghanti et al. 2009), BAP and NAA (Chakraborti et al. 2006; Yadav and Singh 2012) and BAP and IAA (Tripathi et al. 2013). Among a variety of combinations and concentrations of BAP and auxins studied for the multiple shoots were induced from all explants, BAP

Table 3 Effect of BAP in combination with IAA or IBA or NAA on multiple shoot induction from Axillary meristem (AME) and cotyledonary node (CN) chickpea cultivar JAKI 9218

Plant growth regulators (in mgl^{-1})		AME		CN	
		Percentage of response (%)	Mean no. of shoots/explant	Percentage of response (%)	Mean no. of shoots/explant
BAP	IAA				
0.0	0.00	32.82 ± 0.68^{g}	01.82 ± 0.76^{g}	28.46 ± 0.78^{g}	02.26 ± 0.84^{g}
1.0	0.05	62.56 ± 0.38^{cdef}	08.42 ± 0.38^{f}	65.72 ± 0.56^{ef}	06.42 ± 0.66^{ef}
2.0	0.05	69.48 ± 0.70^{ab}	18.56 ± 0.64^{a}	76.58 ± 0.86^{a}	14.26 ± 0.82^{ab}
3.0	0.05	63.54 ± 0.58^{cde}	12.63 ± 0.58^{bcd}	68.72 ± 0.40^{cd}	11.44 ± 0.62^{bcd}
1.0	0.50	62.58 ± 0.40^{cdef}	10.56 ± 0.64^{e}	66.61 ± 0.48^{cde}	08.62 ± 0.56^{cde}
2.0	0.50	67.88 ± 0.42^{ab}	14.38 ± 0.36^{bcd}	72.35 ± 0.66^{bc}	12.56 ± 0.48^{ab}
3.0	0.50	64.76 ± 0.76^{cde}	12.62 ± 0.42^{bcd}	67.34 ± 0.49^{cde}	10.38 ± 0.38^{bcd}
BAP	IBA				
1.0	0.05	80.68 ± 0.18^{bc}	22.28 ± 0.36^{bcd}	74.72 ± 0.68^{bcde}	12.61 ± 0.46^{de}
2.0	0.05	86.78 ± 0.38^{a}	28.34 ± 0.66^{a}	87.64 ± 0.60^{a}	18.47 ± 0.58^{ab}
3.0	0.05	72.28 ± 0.66^{ef}	16.52 ± 0.54^{ef}	75.34 ± 0.47^{bcd}	14.36 ± 0.82^{bc}
1.0	0.50	76.34 ± 0.58^{d}	21.82 ± 0.48^{bcd}	74.44 ± 0.36^{bcde}	13.28 ± 0.64^{cd}
2.0	0.50	79.48 ± 0.69^{bc}	23.68 ± 0.88^{bcd}	76.48 ± 0.80^{bcd}	16.52 ± 0.48^{ab}
3.0	0.50	68.43 ± 0.84^{ef}	14.47 ± 0.36^{ef}	68.28 ± 0.44^{cdef}	10.68 ± 0.56^{ef}
BAP	NAA				
1.0	0.05	73.52 ± 0.84^{c}	14.24 ± 0.52^{cd}	72.02 ± 0.48^{cd}	10.40 ± 0.48^{de}
2.0	0.05	86.62 ± 0.48^{a}	22.85 ± 0.46^{a}	86.48 ± 0.65^{a}	16.62 ± 0.52^{ab}
3.0	0.05	65.52 ± 0.64^{def}	11.43 ± 0.64^{de}	69.27 ± 0.50^{ef}	08.31 ± 0.63^{ef}
1.0	0.50	67.82 ± 0.78^{def}	12.66 ± 0.72^{cd}	73.62 ± 0.66^{bc}	11.74 ± 0.82^{cd}
2.0	0.50	77.86 ± 0.64^{b}	18.20 ± 0.56^{b}	76.48 ± 0.28^{bc}	14.85 ± 0.46^{ab}
3.0	0.50	65.78 ± 0.82^{def}	10.62 ± 0.62^{ef}	70.26 ± 0.82^{de}	12.32 ± 0.54^{cd}

Each experiment was repeated thrice with 30 replications. Values represent the mean ± standard error. Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level

in combination with IBA was an efficient combination for multiple shoot formation in chickpea.

Shoot elongation, rooting and acclimatization

Induced shoot buds into elongated shoots is a critical step in legume regeneration. Several successful plant regeneration protocols have been reported in chickpea, still, it is considered to be highly recalcitrant species (Atif et al. 2013; Leonetti et al. 2018). The multiple shoot buds induced from all four explants were failed elongate on the same media at the end of the second subculture. For the optimization of shoot elongation, different concentrations and combinations of PGRs have evaluated the multiple shoot buds induced from four different explants of chickpea. The four explants with multiple shoot buds were shifted to shoot elongation media (SEM) for 15-d and subcultured for two passages on SEM at 7-d intervals. IAA (0.05 mg l^{-1}) in combination with BAP (1.0 mg l^{-1}) enhanced the elongation of healthy shoots with a higher percentage of response when compared to individual treatment of either BAP or KIN. The maximum percentage of response was 96.68 on medium fortified with IAA (0.05 mg l^{-1}), GA_3 (1.0 mg l^{-1}) and BAP (1.0 mg l^{-1}), with an average shoot length of 8.82 cm. Chakraborti et al. (2006) reported 80% of shoot elongation using half-seed explant of chickpea on medium containing IAA (1.15 µM). Yadav and Singh (2012) observed efficient shoot elongation on medium fortified with BAP (2.0 mg 1^{-1}), NAA (0.6 mg l^{-1}) and GA_3 (1.0 mg l^{-1}) in chickpea. These results suggest that the maximum level of shoot elongation for chickpea can be achieved from multiple shoots induced in different explants on medium supplemented with BAP, IAA, and GA_3.

Rooting of in vitro regenerated chickpea shoots is regarded as one of the most crucial bottlenecks for successful plant regeneration. In the present investigation, three auxins (IAA, IBA, and NAA) were used with two concentrations (1.0 and 2.0 mg l^{-1}) to optimize the rooting efficiency of the chickpea. The rooting frequency was significantly different in various treatments of auxins. A total of 120 well-developed shoots (thirty shoots from each explant type) were transferred to the rooting medium amended various concentrations of auxins. Out of 120 shoots, 118 shoots were successfully rooted (98.33%) to form complete plantlets on the medium amended with IBA (2. 0 mg l^{-1}), whereas 83 shoots

Fig. 2 Plant regeneration from four different explants of chickpea cv. JAKI9218 on MSM5 medium supplemented with BAP (2.0 mg l^{-1}) and IBA (0.05 mg l^{-1}). **a** Multiple shoots from embryo axis explant (inset: explant) after 6 weeks of culture, **b** multiple shoots from half-seed explant (inset: adaxial side of explant) after 6 weeks of culture, **c** multiple shoots from axillary meristem explant (inset: explant) after 6 weeks of culture, **d** multiple shoots from cotyledonary node (inset: explant) explant after 6 weeks of culture, **e** root induction on medium supplemented with IBA (2.0 mg l^{-1}) after 3 weeks of culture, **f** complete plantlet and **g** acclimatized plantlet under greenhouse conditions after 4 weeks

induced roots on NAA containing medium (69.16%) and 41 shoots formed roots on IAA supplemented medium (34.16%) to develop complete plantlets. Among diverse concentrations of three auxins tested, medium supplemented with IBA was found to best followed by NAA for root induction frequency in chickpea. The lowest frequency of root induction was observed on medium augmented with IAA induced one to three small (< 2.5 cm in length) and weak roots along with callus formation. The media augmented with NAA produced three to four roots (> 5.0 cm in length) per shoot. The percentage response of root formation was also consistently higher in the medium containing IBA at 2.0 mg l^{-1}. The average number of roots per shoot was 14.25 cm and an average length was 11.25 cm on medium fortified with IBA at 2.0 mg l^{-1} (Fig. 2e, f). The rooting response was elicited by different auxins (D'Silva and D'Souza 1992), based on their type and level in the medium were found to differ from tissue to tissue and species to species (Arora and Chawla 2005). Among the three auxins tested, IBA was excellent in inducing rooting percentage and number of roots per shoot followed by NAA. IBA has been commonly used for in vitro rooting of chickpea (Jayanand et al. 2003; Anwar et al. 2008; Kumari et al. 2018). In the present investigation, the response, as well as the nature of roots induced from shoots, different from concentrations of IBA and NAA used. Similar observations were also made in earlier reports on the rooting efficiency of IBA followed by NAA in chickpea (Sunil et al. 2015). IAA induced small and weak roots and callus formation at the cut ends of shoots. The presence of callus between the root and shoot resulted in poor vascular development, which made poor survival of the plantlets (Chitra and Padmaja 2005).

The plantlets were observed with the formation of a new shoot (leaves) within 2–3 weeks. The plantlets were

subsequently shifted to pots containing an equal ratio of sterile vermiculite, sand, and soil. A total of 108 plantlets, 32 plantlets from embryo axis, 28 plantlets from half-seed, 22 plantlets form axillary meristem and 26 plantlets from cotyledonary node plantlets were transferred to pots out of which 25 plantlets from embryo axis, 19 plantlets from half-seed, 16 plantlets from axillary meristem and 18 plantlets from cotyledonary node, a total of 78 plantlets were survived under greenhouse conditions (Fig. 2 g). The overall survival rate was recorded as 72% after 4 weeks of transplantation. The in vitro regenerated plantlets from different explants were appeared to be morphologically similar to their mother plants.

Genetic fidelity assessment by using SCoT and ISSR markers

A total of 12 randomly selected plantlets, three from each explant type were subjected to SCoT and ISSR marker analysis to determine the genetic variability. For the better analysis of genetic stability and rejecting the possibility of somaclonal variants, it has always been suggested to use more than one marker (Singh et al. 2013; Rohela et al. 2019). Among the 10 SCoT primers, only four primers were amplified namely SCoT 1, 4, 6, 10 with 13 bands. SCoT 6 has produced six bands in the range 500–3000 bp which were monomorphic (Fig. 3) (Table 4). Amplification with 10 ISSR primers yielded 32 bands in total with 3 as the average number of bands per primer and band size ranging from 300 to 1500 bp (Table 5). ISSR primer 3 provided good amplification and produced 4 DNA bands with distinct and good intensity in the range of 600–1300 bp (Fig. 4). Among different molecular markers, SCoT markers are gaining much attention due to their good quality and better feasibility over other molecular markers in genetic diversity and homogeneity studies (Collard and Mackill 2009; Cabo et al. 2014; Thakur et al. 2016). Genetic stability of in vitro regenerated plants were

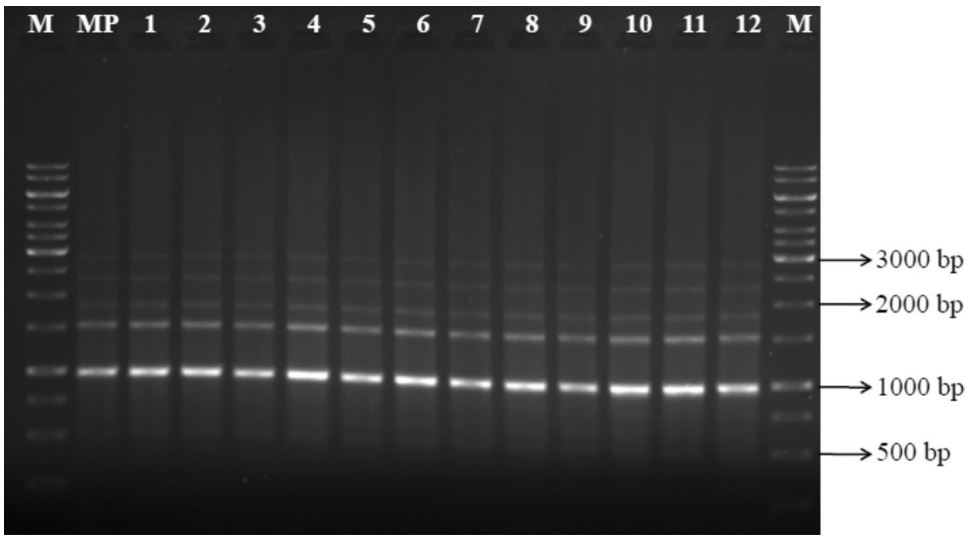

Fig. 3 SCoT profiles of the mother plant and tissue culture raised plantlets of chickpea using SCoT6 primer. Lane M: 1 kb DNA ladder. Lane MP: DNA banding pattern of the mother plant. Lane 1–3: DNA banding pattern of acclimated plants that were raised from embryo axis explants. Lane 4–6: DNA banding pattern of acclimated plants that were raised from half-seed explants. Lane 7–9: DNA banding pattern of acclimated plants that were raised from cotyledonary node explants. Lane 10–12: DNA banding pattern of acclimated plants that were raised from axillary meristem explants

Table 4 List of SCoT primers, their sequence, number and approximate band length of DNA bands

S. No	Primer code	Primer sequence (5′–3′)	Number of bands amplified	Approximate band length (bp)
1	SCoT1	CAACAATGGCTACCACCA	5	600–2000
2	SCoT2	CAACAATGGCTACCACCC	0	–
3	SCoT3	CAACAATGGCTACCACCG	0	–
4	SCoT4	ACCATGGCTACCACCGTC	1	2000
5	SCoT5	ACGACATGGCGACCAACG	0	–
6	SCoT6	ACCATGGCTACCACCGCC	6	500–3000
7	SCoT7	ACGACATGGCGACCACGC	0	–
8	SCoT8	ACGACATGGCGACCGCGA	0	–
9	SCoT9	CCATGGCTACCACCGCAG	0	–
10	SCoT10	GCAACAATGGCTACCACC	1	1800

Table 5 List of ISSR Primers, their sequence, number and approximate band length of DNA bands

S. No	Primer code	Primer sequence (5′–3′)	Number of bands amplified	Approximate band length (bp)
1	ISSR1	AGAGAGAGAGAGAGAGG	7	300–1200
2	ISSR2	GAGAGAGAGAGAGAGAT	4	300–1300
3	ISSR3	GAGAGAGAGAGAGAGAC	4	600–1300
4	ISSR4	TCTCTCTCTCTCTCTCC	2	650–1350
5	ISSR5	AGAGAGAGAGAGAGAGYT	2	600–1250
6	ISSR6	AGAGAGAGAGAGAGAGTC	3	600–1350
7	ISSR7	CACACACACACACACARC	2	500–900
8	ISSR8	ACACACACACACACACT	2	650–1500
9	ISSR9	GAGAGAGAGAGAGAGAYT	5	300–1200
10	ISSR10	TGTGTGTGTGTGTGTGRA	1	1100

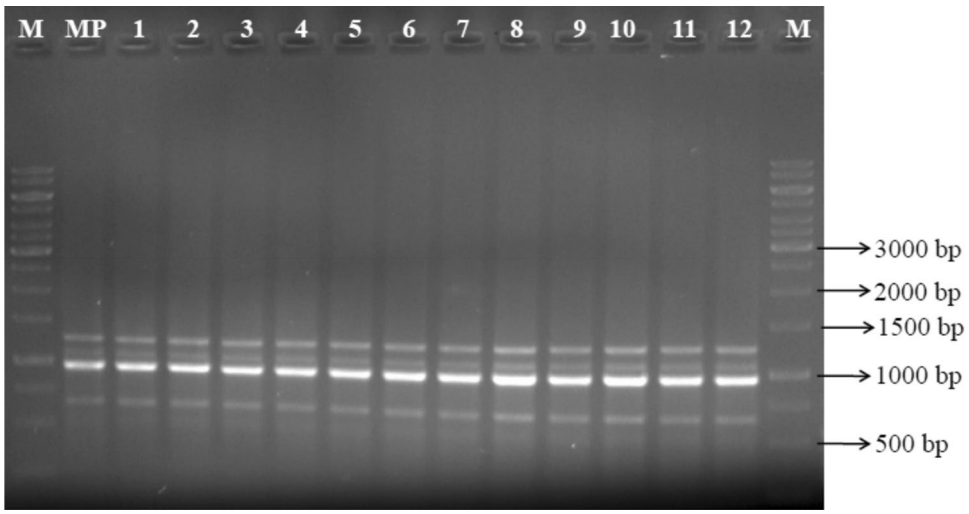

Fig. 4 ISSR profiles of the mother plant and tissue culture raised plantlets of chickpea using ISSR3 primer. Lane M: 1 kb DNA ladder. Lane MP: DNA banding pattern of the mother plant. Lane 1–3: DNA banding pattern of acclimated plants that were raised from embryo axis explants. Lane 4–6: DNA banding pattern of acclimated plants that were raised from half-seed explants. Lane 7–9: DNA banding pattern of acclimated plants that were raised from cotyledonary node explants. Lane 10–12: DNA banding pattern of acclimated plants that were raised from axillary meristem explants

analyzed by SCoT markers reported in several plant species such as *Cleome gynandra* (Rathore et al. 2014), *Citrullus lanatus* (Vasudevan et al. 2017), *Saccharum officinarum* (Sathish et al. 2018), *Sesamum indicum* (Elayaraja et al. 2019), and *Pisum sativum* (Ajithan et al. 2019). Similarly, ISSR primer based assessment of in vitro propagated plants was reported in *Morus* spp (Rohela et al. 2018), *Eleusine coracana* (Babu et al. 2018), *Solanum trilobatum* (Pendli et al. 2019), *Corallocarpus epigaeus* (Vemula et al. 2019), and *Albizia lebbeck* (Saeed et al. 2019). In our study, amplified DNA bands with SCoT and ISSR markers were monomorphic which validates the genetically true-to-type and stable plantlets of chickpea. This is the first report using SCoT and ISSR molecular markers for the evaluation of the genetic homogeneity of micropropagated plants in chickpea.

Conclusions

The present work demonstrates shoot bud induction and plant regeneration from embryo axes, half seed, axillary meristem and cotyledonary node explants of chickpea cultivar JAKI9218. The embryo axes and half-seed explants responded most favourably when compared to axillary meristem and cotyledonary node explants. The synergistic effect of cytokinins with different auxins resulted in an increased percentage of shoot production, elongation and rooting. The protocol enables the generation of a huge number of

regenerated plantlets in a relatively little period. The genetic fidelity of in vitro regenerated plants from four different explants has been confirmed using SCoT and ISSR molecular markers. The procedure described herein will be feasible for chickpea genetic transformation experiments to transfer agronomically novel traits for effective crop improvement.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest in the present study.

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