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Original article

Induction of somatic embryogenesis in *Brassica juncea* L. and analysis of regenerants using ISSR-PCR and flow cytometer

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ABSTRACT

A new and simple protocol has been developed and standardized for direct somatic embryogenesis and plant regeneration from aseptic seedlings derived from immature *Brassica juncea* seeds. Depending on the age of immature seeds and nutrient media, in vitro occurrence of embryogenesis and the number of embryos from each seedling have varied greatly. The largest number of somatic embryos, producing 12.7 embryos per seedlings, have been developed by seedlings obtained from immature seeds collected after 21 days of pollination (DAP). Effect of different nutrient media [Gamborg (B5), Murashige and Skoog (MS) and Linsmaier and Skoog (SH)] and carbon sources (fructose, glucose, maltose and sucrose) were assessed to induce somatic embryos and the maximum response were achieved on Nitsch culture medium fortified with sucrose (3% w/v) followed by fructose and maltose. The somatic embryo converted into complete plantlets within 04-weeks of culture on Nitsch medium containing half-strength of micro and macro salts. The regenerated plantlets were successfully established in soil with 90% survival rate. The acclimated plants were subsequently transferred to field condition where they grew normally without any phenotypic differences. Genetic stability of *B. juncea* plants regenerated from somatic embryos were confirmed by inter-simple sequence repeat (ISSR)-PCR analysis and flow cytometry. No significant difference in ploidy level and ISSR banding pattern were documented between somatic embryo's plants and control plants grown ex vitro.

1. Introduction

Brassica juncea L. (Brassicaceae) is known as Indian or brown mustard, owing to its spicy taste, high content of various bioactive chemical compounds and abundance, it is one of the mustard species commonly used in human food and animal feed. (Okunade et al., 2015). The plants, however, of *B. Juncea* is usually used in the processing of biodiesel and pesticides (Kashyap et al., 2019). It is considered as one of the most valuable oil-seed crops of the *Brassica* genus. *B. juncea* has numerous desired agronomic characteristics, with resistance to disease, tolerance to drought, early maturity, shattering resistance. (Downey, 1990; Woods et al., 1991; Zhang et al., 2018). Successful micropropagation, regeneration and genetic transformation based on different tissue culture techniques has been reported in various *Brassica* species (Mathews et al., 1990; Eapen and George, 1997; Cao and Earle, 2003; Wahlroos et al., 2003; Kashyap et al., 2019).

Somatic embryogenesis (SE) is the utmost significant processes in in vitro reproduction, involving multiple stages from the development in pro-embryogenic growth mass to embryo and plant maturation and proliferation (von Arnold et al., 2002). SE is typically favoured to other methods of production as it can be used in various plant micropropagation, genetic alteration and accelerated proliferation systems. (Arya et al., 1993; Shinjiro et al., 2002; Al Shamari et al., 2018). In principle, where suitable explants are used, all plant species have the competence to produce somatic embryos in vitro by providing suitable nutrient media, growth regulators and culture conditions. (George et al., 2008).

One of the important factors to be evaluated in micropropagated plantlets when a process is going to be used for commercialization is genetic uniformity of regenrants. Inter Simple Sequence Repeat (ISSR) is a technique utilizes microsatellite sequences as polymerase chain reaction (PCR) primers to generate multilocus markers. This technique is very useful in genetic diversity

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examination and evolutionary biology studies (Pradeep Reddy et al., 2002). In *B. juncea*, ISSR-PCR technique was utilized to examine the genetic diversity among the native species in China (Huangfu et al., 2009) and to identify molecular markers related to production of 2-propenyl glucosinolate (Ripley and Roslinsky, 2005). Furthermore, the same technique was applied to examine the genetic diversity among 11 accessions of *B. rapa* var. *chinensis* (Linnaeus) Kitamura (Shen et al., 2016). In addition, flow cytometric analysis (FCA) of plants cells and tissues have been recognized as one the most steadfast method during recent days for estimation of ploidy level, and genome size by calculating the nuclear DNA content. FCA provides unsurpassed ease, speed and accuracy in comparison the traditional method of chromosomal counting (Doležel et al., 2007) and being significantly employed for validation of tissue culture plants including Oak (Endemann et al., 2001), *Larix decidua* (von Aderkas et al., 2003), *Eucalyptus globulus* (Pinto et al., 2004), *Quercus suber* (Loureiro et al., 2005), *Juniperus phoenicea* (Loureiro et al., 2007), and *Solanum lycopersicum* (Alatar et al. 2017) and Brahmi (Faisal et al., 2018). The main objective of this study was to develop an efficient and robust system of direct somatic embryogenesis and plant regeneration from immature seeds of *B. juncea*. Additionally, this work also addressed the genomic stability of plants derived from somatic embryos, compared with control plants grown ex vitro, using flow cytometry and inter-simple sequence repeat PCR analysis (ISSR).

2. Material and methods

2.1. Starting materials and preparation of explants

Fresh and healthy seeds *of Brassica juncea* L. cv. Kala moti were grown in a glass house at 24 ± 2 °C under LED (A light-emitting diode) light with a photoperiod of 16/8 h. After flowering the pollination were performed and age of the seeds were calculated according to the days after pollination (DAP). Green immature seed-pods of various stages (7, 14, 21 and 28 days) were used as explants for further in vitro experiments.

2.2. Induction of somatic embryos

Healthy green pods were surface disinfected with chorine based commercial bleach solution (2.5% (v/v, Clorox Co. Saudi Arabia) containing 0.05% (v/v) Tween 20. With sterile purified water, the pods were washed 4–5 times after 10 min to eliminate all the traces of bleach. Green immature seeds were aseptically isolated from the sterilized pods and planted on different strengths of growth hormones free Nitsch medium (Nitsch and Nitsch, 1969; Duchefa BV, Haarlem, The Netherlands) (pH 5.7) supplied with 3% (w/v) sucrose (LOBA Chemie, Mumbai India) and 0.7% (w/v) agar (SIGMA-ALDRICH, Spain) before being sterilized at 15 psi (121 °C) for 20 min in a steam-based ALP-autoclave (CLG-32L, ALP Co., Tokyo, Japan). All the cultured plates were incubated at 24 ± °C in dark for 48 h and after that they were transferred under light (16/8h light–dark cycle) with luminous intensity of 50 μmol $m^{-2}s^{-1}$ in a growth chamber (Conviron Adaptis, USA) provided by the cool LED lamps.

2.3. Effect of nutrient medium and carbohydrate sources

Different nutrient compositions have also been studied and compared to achieve the optimum response from seedlings of immature green seeds for in vitro somatic embryogenesis. For the development of a standardized process for in vitro somatic embryogenesis, the other media like MS (Murashige and Skoog, 1962; Duchefa BV, Haarlem, The Netherlands), B5 (Gamborg et al., 1968; Duchefa BV, Haarlem, The Netherlands), and SH (Schenk and Hildebrandt, 1972; Duchefa BV, Haarlem, The Netherlands) were used together with the Nitsch medium. In addition, outcome of other carbohydrate sources viz. fructose, glucose and maltose were also tested and compared with sucrose for direct somatic embryogenesis by supplementing them in -Nitsch medium.

2.4. Embryos maturation and plant development

Following initiation of somatic embryogenesis, the somatic embryos of *B. juncea* derived from immature green seeds were moved to fresh Nitsch medium with half-strength of micro and macro salts for further growth, germination and seedling formation. The full plantlets raised from the somatic embryos were moved to sterile vermiculite-containing plastic pots and watered twice a week with 1⁄4-strength Nitsch salt solutions. To maintain the high humidity, planted plants were first covered with clear bags and eventually released over a span of 2 weeks to expose them to ambient humidity. After one month the acclimated plants developed in vitro from the somatic embryos of *B. juncea* were transferred to normal condition.

2.5. Flow cytometric analysis of plantlets

Flow cytometric histogram was produced by running the nuclei extracted from 100 mg leaf tissues of regenerated plants and ex vitro cultivated plants chopped in isolation buffer, as defined by Galbraith et al. (1983), for the assessment of ploidy. After filtration with a double-layered nylon film of 0.22 μM, the nuclei suspension were stained for 10 min with 50 $\mu g ml^{-1}$ of PI (Propridium iodide, Sigma, USA) for 10 min and analyzed using flow cytometry equipped with System II Program, Version 3.0 (Coulter ® Epics XL-MCLTM, Beckman Coulter Inc., California, USA).

2.6. ISSR analysis of plantlets

Genetic stability of embryos derived plantlets was assessed using ISSR markers and compared to plantlets grown ex vitro. Genomic DNAs were isolated from the leaf tissues of randomly selected plantlets using DNeasy® Plant Mini Kit (Qiagen Cat No.# 69104). NanoDrop™ 2000c Spectrophotometers (ThermoFisher Scientific™, USA) used to perform the purity and quantification of isolated leaf DNA's. 10 sets of ISSR primer synthesized from Gene Link™ (Gene Link, NY, USA) were screened and used to determine the uniformity of the plantlets by performing the polymerase chain reaction (PCR) in a thermal cycler gradient (BIORAD T100™, Bio-Rad Laboratories, USA). 20 μl of PCR reaction mixture were used containing 2.0 μl of Taq buffer (10X) with KCL (Thermo Scientific™), 1.2 μl of 25 mM $MgCl_2$ (Thermo Scientific™), 0.4 μl of 10 mM dNTPs (Thermo Scientific™), 0.2 μl Taq polymerase recombinant (Thermo Scientific™) 1.0 μl of ISSR primers (Gene Link™, USA) and 25 ng template DNA. For amplification a program in the PCR machine were set for 45 cycles with DNA denaturation at 94 °C of 5 min, an annealing at 35 °C for 1 min and 1 min at 72 °C for extension followed by a final extension for 10 min at 72 °C. The amplified PCR products were separated in horizontal electrophoresis (Horizon 14.11 Biometra Analytik Jena AG, Germany) using 1.2% (w/v) agarose-gel made in TAE buffer (1X, Tris-acetate-EDTA, pH 8.0) comprising 5.0 μl ethidium bromide The electrophoresis unit were run for 2 h at 50 V and the image of the gel were captured and analyzed using UV gel imaging system (G:Box F3, Syngene, UK). For analysis well depicted and reproducible DNA bands were scored.

3. Results

In the first set of experiments we investigate the morphogenic ability of aseptic seedlings to develop somatic embryos in vitro. Immature seeds (7, 14, 21 and 28 days old) of *B. juncea* cultured on Nitsch medium start germinating after 3–4 days of planting. The hypocotyls of the germinating seedlings swollen, and somatic embryos were directly initiated from the region after 3 weeks of culture. Frequency of somatic embryogenesis and number of embryos per seedling were varied considerably according to the green pods collected from the plants (Fig. 1A). Highest number of embryos were recorded from immature seeds collected after 21 days of pollination and produced 12.7 embryos per seedling. While the immature seeds collected after 07 days of pollination produced the lowest number of embryos with 4.1 embryos per seedling.

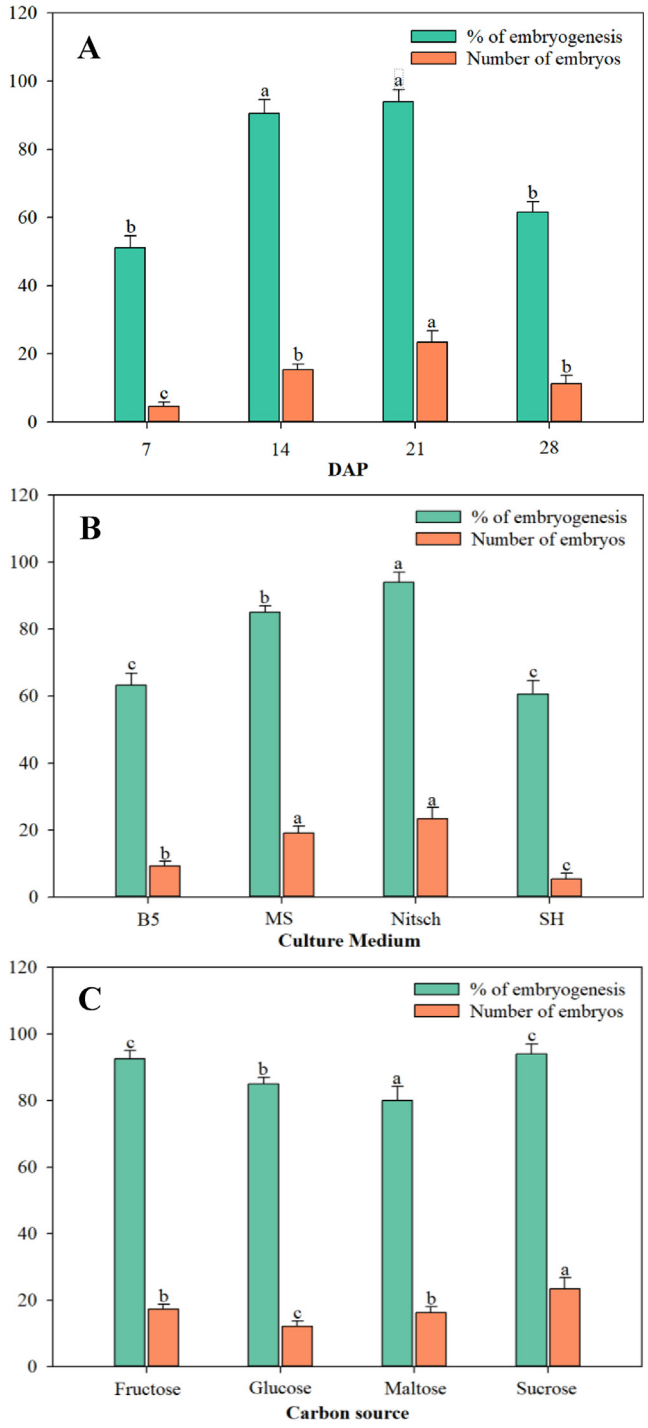

Fig. 1. Impact of DAP (A), medium (B) and carbon sources (C) on in vitro embryogenesis from immature seeds of *Brassica juncea*. Within the group, graph-bars denoted by the small alphabet's letters are not statistically different.

In addition, different nutrient media, B5, SH and MS, were analyzed in order to generate a sufficient number of *B. juncea* somatic embryos from immature seeds. (Fig. 1B). Among some of the different culture media used in this investigation, the highest number of embryos were obtained from seedlings derived from 21 days old immature green seeds on Nitsch medium. While the SH media produced the lowest frequency and number of somatic embryos.

In this investigation effects of various carbon sources, including fructose, glucose, maltose, and sucrose, on somatic embryogenesis in *B. juncea* was also evaluated in this study (Fig. 1C). Nitsch media enriched with sucrose was found to repeatedly produce highly embryogenic colonies, with a higher proportion of somatic embryos compared to media supplemented with glucose, fructose, or maltose. There were substantial variations between all carbon sources in the percentage of somatic embryogenesis and mean number embryos ($p = 0.5$), with the exception of maltose and glucose (Fig. 1C). Data obtained in this investigation showed that sucrose was the best for induction of somatic embryos from the seedlings derived from immature zygotic embryos.

Repetitive somatic embryogenesis (secondary embryos) were perceived on the same induction medium from the primary embryos of *B. juncea*. The induced embryos were moved to growth-regulators free half-strength Nitsch medium containing 3% sucrose after two weeks, where they transformed within 4 weeks into complete plantlets with an average germination rate of 90–95%. *B. juncea* somatic embryos of different developmental stages are depicted in Fig. 2. In this investigation the phases of embryogenesis were asynchronous, therefore the in vitro-plantlets obtained from the same culture medium were at various stages of development. With a 90% survival rate, plantlets with a well-developed root and shoot system were successfully transferred to the soil. All habituated *B. juncea* plants were eventually transported to the field and grew gradually without any phenotype difference in the natural state of the climate.

One of the most important prerequisites for the effectiveness of any in vitro protocol is the genetic integrity of plants for mass propagation and commercialization. We used the directed mini-satellite region amplification (ISSR) and flow cytometry to check out the genetic uniformity of somatic embryo-derived plants of *B. juncea* and compared with the normal field grown plants. Patterns of ISSR molecular markers were used to evaluate and compare the in vitro derived plants with the field grown plants. 10 ISSR DNA-oligos were used for PCR amplification, and an average of 11.1 bands per primer were formed (Table 1). Only the clear and reproducible band were counted and compared among the replicates and found that all the ISSR bands were monomorphic in nature (Fig. 3). In the present investigation, the ploidy level of nuclei isolated from embryo-derived plants, as well as control plants grown to ex vitro conditions ascertain by using flow cytometry. Comparison of fluorescence peaks in the histograms generated from PI-stained nuclei showing unimodal peak of the nDNA (nuclear-DNA) content corresponding to 2x from the nuclei of ex vitro grown plants thereby substantiating that there was no variation in ploidy levels as well as nDNA content (Fig. 4). In this study there were no substantial variations in their usual fluorescence peak positions in contrast to the fluorescence peaks in histograms obtained from the flow cytometry study validating that there was no difference between in vitro somatic embryo-derived plants of *B. juncea* and control plants grown to ex vitro conditions.

Fig. 2. Different developmental stages of somatic embryos-derived from germinating immature seeds of *Brassica juncea.*

Table 1
ISSR primers screening to evaluate the extent of genetic reliability of plantlets derived from immature seeds *Brassica juncea.*

S. No.	ISSR Oligos	Oligo sequences (5′–3)	T_a (°C)	Amplified bands
1.	GL825	ACACACACACACACA CT	45.0	11
2.	GL827	ACACACACACACACACG	49.7	10
3.	GL841	GAGAGAGAGAGAGAGAYC	49.7	9
4.	GL855	ACACACACACACACACYT	49.7	15
5.	GL866	CTCCTC CTCCTCCTCCTC	55.9	19
6.	GL868	GAAGAAGAAGAAGAAGAA	45.0	13
7.	GL880	GGGTGGGGTGGGGTG	49.7	15
8.	GL889	DBDACACACACACACAC	45.0	12
9.	GL891	HVHTGTGTGTGTGTGTG	45.0	7
10.	GL900	ACTTCCCCACAGGTTAACACA	57.8	0
Total number of bands =				111
Average number of bands/primers				11.1

T_a = Annealing temperature.

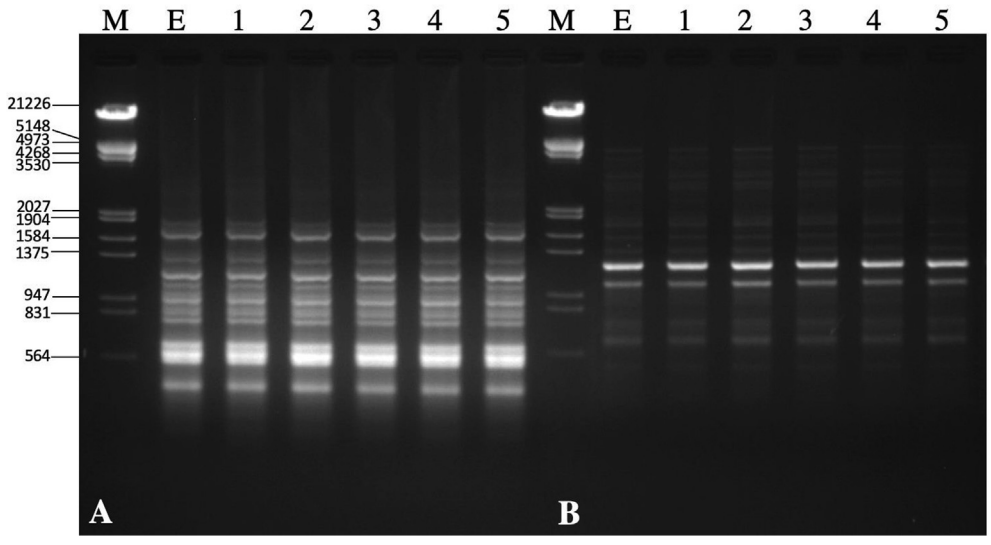

Fig. 3. ISSR analysis of *Brassica juncea* plants. A generated from ISSR primer GL-855; B generated from primer GL-880. M = Molecular weight marker; E = Control plants grown ex vitro; 1–5 = Plants derived in vitro from somatic embryos.

4. Discussion

Embryogenesis is the mechanism by which, either from a zygote or from somatic cells, embryo development is started. Embryogenesis, in planta, is divided into two different phases; early morphogenesis that give birth to embryonic form of cells such as tissues, and organ systems, and advanced developmental stages that cause the embryo to reach the physiological state of desiccation and quiescence (West and Harada, 1993). The most important in vitro reproduction system is somatic embryogenesis (SE), involving many stages from proembryogenic growth mass formation to embryo and plant development and propagation

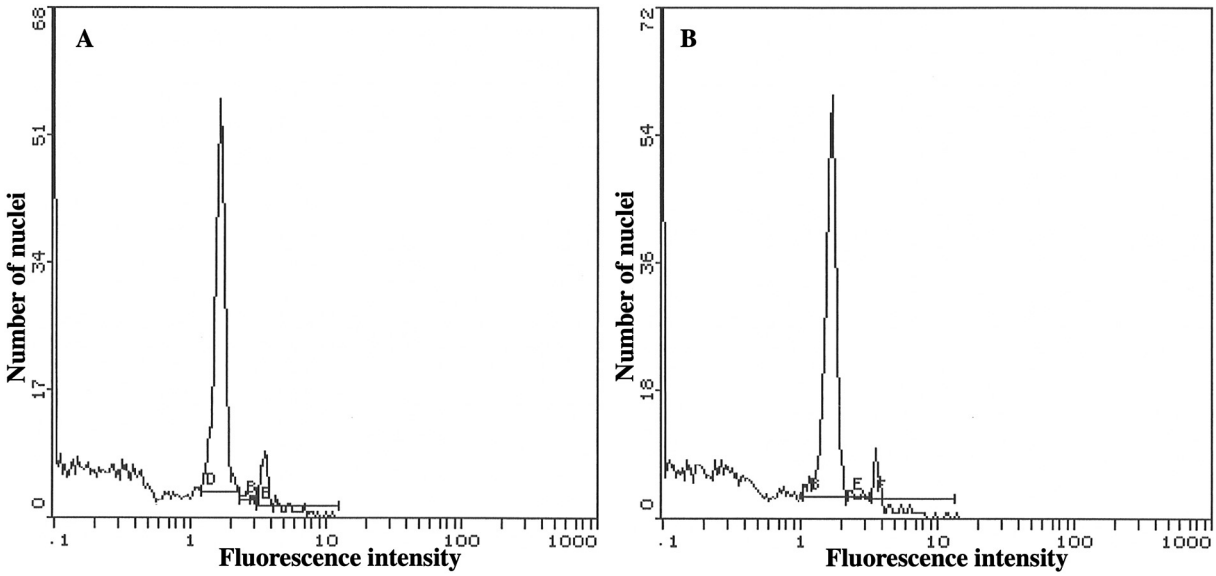

Fig. 4. Flow cytometric histograms generated from PI stained nuclei of *Brassica juncea* plants. A. Control plants grown ex vitro; B. Plants derived in vitro from somatic embryos.

(von Arnold et al., 2002). SE is usually preferred over the methods of reproduction, as it can be used in diverse schemes of plant propagation in vitro with increased proliferation and genetic modification. (Arya et al. 1993; 2002, Shinjiro et al. 2002; Al Shamari et al. 2018). In general, all plant species have the ability to develop somatic embryos if sufficient explants are used, by having adequate nutritional material, growth regulators and culture conditions, and the mature (or dormant) embryos germinates upon receiving correct signal and produce seedlings. In this study morphogenetic potential of seedlings explants derived from immature seeds were evaluated for producing somatic embryos. Immature zygotic embryos have been documented to have a substantially greater embryogenic ability in rapeseed than mature embryos (Koh and Loh, 2000; Burbulis et al., 2007). In certain crops, embryonic zygotic embryos possess the capacity to induce somatic embryos while mature zygotic embryos lack the ability (Raemakers et al., 1995).

The effect of different growth regulator free media recipes observed in this study contrasts with the effect documented in immature zygotic embryo cultures of various species for somatic embryogenesis, including *Ginkgo biloba* (Laurain et al., 1996) and *Rosa hybrida* (Kim et al., 2003) where exogenous cytokinin is necessary in the medium to induced to somatic embryos. In plant life, carbohydrates are the building blocks of macromolecules that play an indispensable role in developmental processes and gene expression in higher plants (Smeekens, 2000; Gibson, 2005). In this study different carbohydrate such as fructose, glucose, maltose, and sucrose were tested for somatic embryogenesis and found that that Nitsch media supplemented with sucrose has been shown to consistently develop highly embryogenic culture. The results are in agreement with an earlier investigation by Ślesak and Przywara (2003) in *Brassica napus* where sucrose was also most effective carbon sources followed by maltose and glucose. Similarly, sucrose was found superior among the tested carbohydrates for optimum embryo induction with uniform developmental stages (Gerdakaneh et al., 2009). Glucose, on the other hand, was found most important for in vitro embryogenesis of four different Cocao genotypes (Traore and Guiltinan, 2006). Whereas, for successful in vitro morphogenic replay and somatic embryogenesis in seedless grapes, glucose and fructose together are required in the medium (Yancheva and Roichev, 2005).

One of the most important prerequisites for the effectiveness of any in vitro protocol is the genetic integrity of plants for mass propagation and commercialization. In this study the embryo derived plantlets were authenticated by inter-simple sequence repeat markers (ISSR) and flow cytometer. The ISSR analysis are in consistent with some earlier findings of evaluation of genetic uniformity based on DNA based molecular markers (Hu et al., 2011; Rathore et al., 2011; Faisal et al., 2018). Flow cytometry has been shown to be a reliable technique in recent years for determining the ploidy and clonal integrity of in vitro-raised plantlets of *Anthurium andreanum* (Gantait and Sinniah, 2011), *Pongamia pinnata* (Choudhury et al., 2014), *Mentha arvensis* (Faisal et al., 2014), *Bacopa monnieri* (Faisal et al., 2018). No major differences in the PI-stained nuclei fluorescence peak derived from young leaves of *B. juncea* plantlets relative to PI-stained nuclei from the control plants were found in this analysis. The results of this analysis are closely associated with some previous studies on *Solanum lycopersicum* (Alatar et al. 2017), *Puya berteroniana* (Viehmannova et al., 2016), *Cucumis melo* (Raji et al., 2018), *Carum copticum* (Niazian et al., 2017), *Bacopa monnieri* (Faisal et al., 2018), and *Juglans regia* (Sadat-Hosseini et al., 2019).

5. Conclusion

In conclusion, an effective method of inducing somatic embryos from seedlings derived from immature *B. juncea* seeds has been developed. Genetic integrity of embryos derived in vitro plants was determined by ISSR markers and flow cytometer to ensure the supply of homogeneous population of *B. juncea*. The developed protocol might be useful for future morphogenetic studies and in vitro manipulation and gene transformation of this crop for higher biomass, oil yield and other agronomic traits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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