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## Original article

Feasible regeneration and agro bacterium-mediated transformation of *Brassica juncea* with *Euonymus alatus* diacylglycerol acetyltransferase (EaDACT) geneIjaz Naeem<sup>a,d</sup>, Iqbal Munir<sup>a,\*</sup>, Timothy P. Durrett<sup>b</sup>, Aqib Iqbal<sup>a</sup>, Karanbir S Aulakh<sup>b</sup>, Mian Afaq Ahmad<sup>a</sup>, Hayat Khan<sup>c</sup>, Imtiaz Ali Khan<sup>e</sup>, Firasat Hussain<sup>c,f</sup>, Muhammad Shuaib<sup>g,\*</sup>, Asad Ali Shah<sup>h</sup>, Ikram Muhammad<sup>i</sup>, Saraj Bahadur<sup>j</sup>, Khaist Begim<sup>k</sup>, Fida Hussain<sup>l</sup><sup>a</sup> Institute of Biotechnology Genetic Engineering, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa 25130, Pakistan<sup>b</sup> Department of Biochemistry and Molecular Biophysics, Kansas State University, 66506, USA<sup>c</sup> Department of Microbiology, University of Swabi, Khyber Pakhtunkhwa 23561, Pakistan<sup>d</sup> Department of Biotechnology, University of Swabi, Khyber Pakhtunkhwa 23561, Pakistan<sup>e</sup> Department of Entomology, The University of Agriculture, Peshawar 25000, Pakistan<sup>f</sup> Department of Microbiology, Cholistan University of Veterinary & Animal Sciences, Bahawalpur 63100, Pakistan<sup>g</sup> School of Ecology and Environmental Science, Yunnan University, Kunming, China<sup>h</sup> Department of Medical Laboratory Technology, College of Applied Medical Science, Jazan University, Saudi Arabia<sup>i</sup> Laboratory of Plant Metabolic Engineering, Faculty of Life Science and Technology, Kunming University of Science and Technology, China<sup>j</sup> College of Life and Pharmaceutical Sciences, Hainan University Haikou China<sup>k</sup> Department of Genetics, Hazara University, Pakistan<sup>l</sup> Department of Botany, Qurtuba University Peshawar, Pakistan

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## ABSTRACT

In the present study an effort has been made to optimize the *in vitro* regeneration protocol for *Agrobacterium*-mediated transformation of *Brassica juncea*, because of its importance as oilseed crops. The highest callus induction frequency of 87% was observed on MS (Murashige and Skoog, 1962) medium supplemented with 4  $\mu$ M 6-benzyladenine (BA) after four weeks of culture period. Subculturing of organogenic calli in MS media with a similar hormonal composition resulted in shoot organogenesis after six weeks of culture cultivation. The highest shoot induction frequency (92%) was recorded on MS medium containing 4  $\mu$ M BA in combination with 1  $\mu$ M of  $\alpha$ -naphthalene acetic acid (NAA). Further, well-developed roots were formed in MS media augmented with 6  $\mu$ M of Indole acetic acid (IAA) in combination with 1  $\mu$ M Kinetin (Kn). Cotyledon explants were exploited *in vitro* for the successful transformation of *B. juncea*. A binary vector comprised of the *Euonymus alatus* diacylglycerol acetyltransferase (EaDACT) gene under the transcriptional control of a glycinin promoter and with a basta selection marker was introduced into *A. tumefaciens* strain GV3101 via electroporation. EaDACT gene is responsible for unusual triacylglycerol's production where the sn-3 position is esterified with acetate instead of the long-chain fatty acid found in the triacylglycerol's. The highest regeneration frequency (100%) of transgenic shoots was observed on MS medium supplemented with 4  $\mu$ M BA plus 1  $\mu$ M NAA in the presence of 25 mg l<sup>-1</sup>

**Abbreviations:** MS, Murashige and Skoog; PGR, plant growth regulator; NAA,  $\alpha$ -naphthalene acetic acid; YEP, yeast extract-peptone medium; PCR, polymerized chain reaction.

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basta and 160 mg l<sup>-1</sup> timintin. The efficiency of stable transformation was found to be approximately 7% in the transgenic plants. Moreover, the transformed regenerated shoots were confirmed by PCR analysis using *EaDAct* gene-specific primers.

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## 1. Introduction

Today energy is the foremost important factor influencing the progress, development, economic growth, social welfare and quality of life in any country (Singh and Ma, 2006). Fossil fuels have been used conventionally as the major energy source, but many countries are often not available domestically in sufficient quantities to meet fast-growing energy demands. Most developing countries spend a considerable amount of hard-earned foreign currency revenues for the purchase of petroleum products to meet their increasing energy demands (Agarwal et al., 2015). The maximum utilization of petroleum products has thus caused a tremendous increase in their prices worldwide. This has led to a search for alternative options, such as the replacement of fossil fuel with other substitutes such as biodiesel (Karmee and Chadha, 2005).

Biodiesel is a renewable transport fuel created from oil extracted from plant seeds or other parts. It is simple to use, biodegradable, non-toxic and eco-friendly (Srivastava and Prasad, 2000). Due to many molecular similarities to the paraffinic diesel, biodiesel can fulfill the demands that diesel engine makes of its fuel. For instance, no engine modifications are required to substitute biodiesel as diesel fuel that can maintain the engine performance. Further, biodiesel is better than diesel in terms of sulphur content, flash point, aromatic content and biodegradability (Slabaugh et al., 1997). *Brassica juncea* of the Brassicaceae family is among the most vital and cultivable plant varieties for seed oil production. It is cultivated in numerous countries of the world including Canada, Australia, China, India and Russia (Rabbani et al., 1998; Woods et al., 1991). This plant and some other species of the family Brassicaceae are medicinally important and contain some important phytochemicals such as vitamins, minerals and anti-carcinogenic agents, etc. (Nosheen et al., 2016; Bahadur et al., 2018a; Ullah et al., 2018a, 2018b; Ashfaq et al., 2019a; Shuaib et al., 2019; Zaman et al., 2019). The potential for production of higher yields in terms of biomass under extreme conditions makes *B. Juncea* an attractive crop for biodiesel production. However, the seed oil produced from this species has the disadvantage of being highly viscous. Due to the presence of high amount of long chain fatty acids, the oil cannot be used directly in diesel engines and must be first trans-esterified to reduce its viscosity (Kulkarni and Dalai, 2006). Poor fuel atomization in contemporary diesel engines occurs due to the high viscosity of plant oils, therefore plant oils with low viscosity can be produced by altering the fatty acid composition of triacylglycerols (TAGs) in vegetable oils (Durrett et al., 2010). Recently, a novel approach has been employed to lower the viscosity of triacylglycerols by acetylating the *sn*-3 position of the long chain of triacylglycerols in vegetable oils, potentially eliminating the requirement of transesterification in-engine use. The seeds of *Euonymus alatus* produce an unusual 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAG), where the *sn*-3 position is esterified with acetate instead of the long-chain fatty acid. The *sn*-3 position of the acetyl group changes the physical and chemical properties of acetyl-TAGs, by reducing their viscosity by approximately 30% compared to regular triacylglycerols (here referred to as long-chain TAGs or lCTAGs, to emphasize the different in the acyl character of the *sn*-3 position of the molecule). A membrane-bound *O*-acyltransferase (MBOAT) enzyme present in the endosperm of *E. alatus* is mainly responsible for the production

of acetyl-TAG. The gene for the expression of this enzyme was sequenced and was named *E. alatus* diacylglycerol acetyltransferase (*EaDAct*). The previous study shows that down-regulation of the DGAT1 pathway for synthesis of lCTAGs leads to the enhanced accumulation of acetyl-TAGs in *Camelina* and *Arabidopsis*. The effective application of this approach to the oilseed crop *Camelina sativa* resulted in up to 85 mol% acetyl-TAGs in the oil, representing the maximum levels of unusual lipids achieved in transgenic plants (Liu et al., 2015).

Thus, the other oil-producing crops can be transformed with the *EaDAct* gene so that they can produce these unusual acetyl-TAGs which possess biofuel and other applications. In addition, genetic engineering will serve to modify field crops with desirable qualitative and agronomic traits. Establishment of an efficient tissue culture system is vital for the success of any plant genetic engineering procedure, especially for *Agrobacterium*-mediated transformation (Liu et al., 2015). With this in mind, the present study was aimed to develop and standardize an efficient and high-frequency *Agrobacterium*-mediated transformation system for *B. juncea* (Raya Anmol) with the *EaDAct* gene.

## 2. Materials and methods

### 2.1. Plant material and in vitro seed germination

Mature and viable seeds of *Brassica juncea* (genotype Raya Anmol) were rinsed quickly with 70% (v/v) ethanol for 5 min prior to surface sterilization with 0.1% (w/v) freshly prepared mercuric chloride solution (HgCl<sub>2</sub>) for 3 min. After rinsing three times with sterile distilled water, all the seeds were placed on MS medium (Murashige and Skoog, 1962) solidified with 0.8% agar in sterilized magenta jars for germination.

### 2.2. In vitro regeneration

Cotyledon explants (~5 mm) were excised from two week old seedlings and were surface sterilized. All the explants were then placed on MS media containing 4.0% (w/v) sucrose and 0.8% (w/v) plant agar in 150 ml conical flask supplemented with various concentrations (2, 4, 6, 8 or 10 μM) of 6-benzyladenine (BA) or in combinations with (1 μM) α-naphthalene acetic acid (NAA) or (1 μM) gibberellic acid (GA<sub>3</sub>). The pH of media was adjusted to 5.8 prior to autoclaving (121 °C, 20 min at 1 atm.). All cultures were incubated in a growth chamber at 16 h photoperiod with a light irradiance of ~40 μmol m<sup>-2</sup> sec<sup>-1</sup>, and temperature was maintained at 25 ± 1 °C with 70% relative humidity (Bangash et al., 2013). In all experiments, PGR free solid MS medium was used as a control treatment. After four weeks of culture period, the callus induction frequency was recorded. Calli were then transferred to fresh medium supplemented with the same plant growth hormones for shoot organogenesis with five replications. After four weeks of culture cultivation the data was recorded as shoot induction frequency (%), number of shoots and shoot length (cm). Long and healthy green shoots were then transferred to rooting medium (20% sucrose and 8% agar) supplemented with (2, 4, 6, 8 or 10 μM) of Indole acetic acid (IAA) alone or in combination with 1 μM Kinetin (Kn).

### 2.3. Plant transformation

#### 2.3.1. *Agrobacterium tumefaciens* strain and binary vector

The *Agrobacterium tumefaciens* strain GV3101 harboring the binary vector plasmid pBinGlyBar4-EaDAcT (Durrett et al., 2010) was used for the transformation of *Brassica juncea*. To construct this vector, the EaDAcT coding sequence was amplified with the primers 5'-ACCAATTGATGATGGATGCTCATCAAGAG-3' and 5'-AGACCTGCAGGTTAAGCGTAATCTGGAACATC-3' which containing MfeI and SbfI recognition sites, respectively (Durrett et al., 2010). Standard restriction enzyme digests followed by ligation allowed the insertion of the PCR amplified fragment into the MfeI and SbfI sites in the multiple cloning site of pBinGlyBar4. This binary vector contains the glycinin 1 promoter from soybean that provides seed-specific expression of coding sequences inserted into the multiple cloning site. In addition, pBinGlyBar4 contains the bar gene which provides resistance against the herbicide glufosinate (phosphinothricin) allowing for the selection of transgenic plants. After sequencing to confirm the fidelity of the inserted sequence, the completed vector was then introduced into *A. tumefaciens* strain GV3101 by electroporation (1.8 kV, 200 Ω, 50 mF).

### 2.4. Co-cultivation medium

The infected *B. juncea* explants were co-cultivated on MS media containing 20 mg l<sup>-1</sup> sucrose, 20X iron sources, 0.1 mg l<sup>-1</sup> myo-inositol and supplemented with 6 μM BA in combination with 4 μM NAA.

### 2.5. Selection media

After co-cultivation the putative transgenic *B. juncea* explants were transferred to MS media containing 20 mg l<sup>-1</sup> basta, 160 mg l<sup>-1</sup> timintin fortified with 4 μM BAP plus 1 μM NAA for the selection of explants (selection medium 1). For efficient selection, the putative transgenic explants were further tested at higher concentrations of basta (25 mg l<sup>-1</sup>) and Timintin (160 mg l<sup>-1</sup>) (selection medium 2).

### 2.6. Rooting medium

The putative transgenic shoots were transferred to rooting medium i.e. MS medium containing 6 μM IAA in combination with 1 μM Kn (Bano et al., 2010).

### 2.7. Preparation of the *Agrobacterium* inoculum

The transformed *Agrobacterium tumefaciens* was cultured overnight at 28 °C on a rotatory shaker in YEP medium containing 50 mg l<sup>-1</sup> kanamycin sulphate, 50 mg l<sup>-1</sup> gentamycin and 25 mg l<sup>-1</sup> rifampicin. The bacterial suspension was then centrifuged at 3000 rpm for 10 min, the supernatant was decanted and bacterial pellet was re-suspended in plan MS medium. The resulting suspension was diluted to obtain an OD<sub>600</sub> of 0.5 which was then used for the transformation experiments.

### 2.8. Explant isolation, inoculation and cultivation

Cotyledons were excised from two weeks old seedlings and were subsequently placed on the cultivation medium followed by inoculation through dipping in the transformed *Agrobacterium* suspension. The plates were sealed with micropore tape and were then transferred to the growth chamber and incubated at 21 °C under 16-hour day length for three days (Mollika et al., 2011).

### 2.9. Selection

After three days on the co-cultivation medium, the explants were transferred to the selection medium and again transferred to the growth chamber. In each step, a control plate was also used with explants not treated with the *Agrobacterium* suspension. After three weeks of incubation, the explants were then directly transferred to a fresh MS media in a 250 ml flask containing 25 mg l<sup>-1</sup> basta. The transgenic shoots were then isolated and transferred to rooting media comprised of 6 μM IAA combined with 1 μM Kn.

### 2.10. Transplantation of plantlets into soil

The rooted plantlets were then shifted to plastic pots containing soil, horticulture and vermiculite at ratio 4:2:1 along with osmotic covered with transparent plastic and placed in an acclimatization room at 21 °C with 70–90% humidity. The temperature of the room was gradually increased up to 25 °C. The transparent cover was removed after 3 weeks for proper hardening to obtain established transgenic plants (Barfield and Pua, 1991).

### 2.11. Molecular analysis of transgenic plants

Genomic DNA was extracted from basta-resistant and control plants using the cetyltrimethyl ammonium bromide (CTAB) method (17). Putatively transformed plants were genotyped for EaDAcT using PCR. The PCR reaction was carried out in a 20 μl mixture containing 50 ng genomic DNA, 200 μM dNTPs, 0.4 μM of each EaDAcT specific primer (Table 1), 2.5 mM MgCl<sub>2</sub>, 2 μl Taq polymerase, buffer (10X) and 2.5 units Taq polymerase enzyme (Thermoscientific®, USA) and PCR grade water for final volume adjustment. The mixture at first was heated at 95 °C for the complete denaturation of genomic DNA and then subjected to amplification in a 30 cycled reaction with the thermal profile of 94 °C for 30 sec, 57 °C for 30 s and 72 °C for 1 min respectively. A final extension at 72 °C was made for 5 min at the end of the reaction.

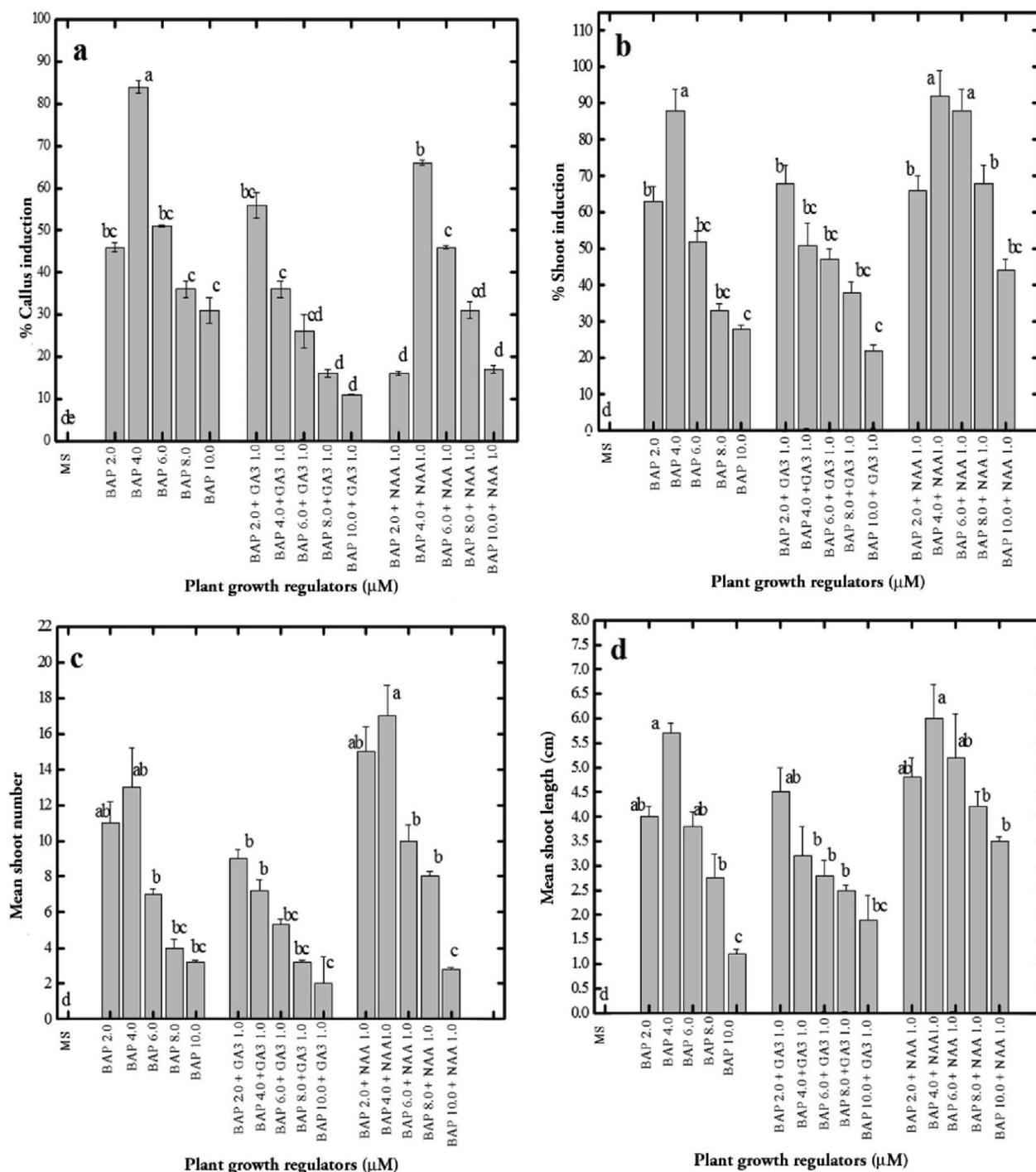
## 3. Results and discussion

### 3.1. Callogenesis

The highest callus induction frequency (87%) was observed when cotyledon explants were incubated on MS medium supplemented with 4 μM BAP (Fig. 1a). The callus formation was initiated at the cut end of explants on day 12th of culture inoculation. Moreover, the callus tissue was compact in texture and yellowish-green in color (Fig. 5a). It was observed that an increase in BAP concentration beyond this optimal level decreased the frequency of callus

**Table 1**  
Primers used for the PCR amplification of target EaDAcT gene in the putative transformed *Brassica juncea*.

Name	Sequence	GC Contents	Tm (°C)	Expected size
EaDAcT-F	ACCAATTGATGATGGATGCTCATCAAGAG	44%	58.9	1 KB
EaDAcT-R	AGACCTGCAGGTTAAGCGTAATCTGGAACATC	47%	68.9	



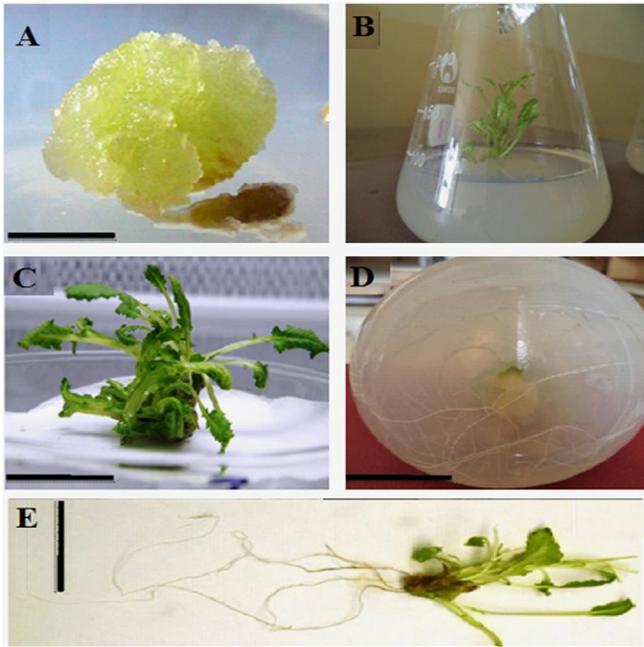
**Fig. 1.** Application of different PGRs concentrations and their impacts on callus formation frequency in *B. juncea*. Values are mean of five replicates with  $\pm$ SE and observations were recorded after 4 weeks of culture. Columns with common alphabets are not significantly different at  $p \leq 0.05$ .

formation. For example, a frequency of only 30% was observed with 10  $\mu$ M BAP (Fig. 1c). Combinations of BAP either with 1  $\mu$ M GA<sub>3</sub> promoted callus formation more effectively when the BAP was applied at lower levels. Explants failed to form callus in plain MS medium (MS medium without growth regulators; used as control). Similarly (Parveen and Shahzad, 2011), observed the highest callogenic response in *Acacia sinuate* cotyledon explants at 4  $\mu$ M BAP. Previously, a combination of 1 mg l<sup>-1</sup> NAA and 1 mg l<sup>-1</sup> GA<sub>3</sub> resulted in 75.3% callus formation from cotyledonary explants of *Sinapis alba* (Abbasi et al., 2011). In contrast, previous work found that callus induction ability in *Brassica napus* cultivars was

enhanced by using 2,4-D in combination with GA<sub>3</sub> or AgNO<sub>3</sub> (Ali et al., 2007).

### 3.2. Organogenesis

The organogenic callus tissue was refreshed on MS medium with similar composition and concentrations of PGRs. Subsequent shoot induction started after 28 days of culture time. A maximum frequency of shoot organogenesis (88%) was observed when callus was refreshed on MS medium supplemented with 4  $\mu$ M BAP (Fig. 2a). Further increases in shoot induction frequency (92%)



**Fig. 2.** Impacts of BAP, NAA and GA<sub>3</sub> on organogenesis in *B. juncea*. Values are mean of five replicates with ±SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are not significantly different at  $p \leq 0.5$ .

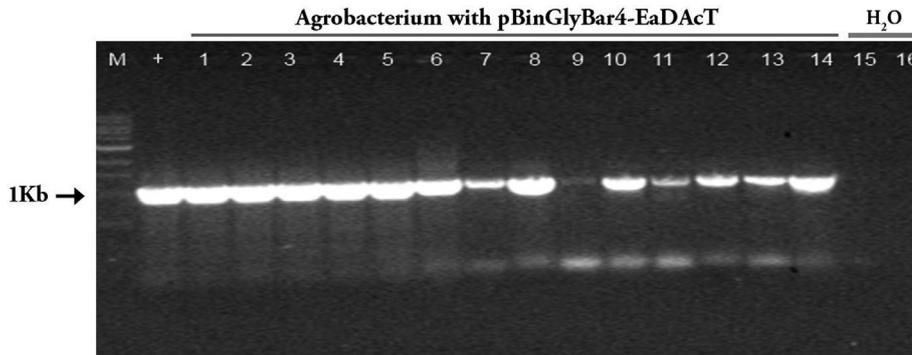
was observed when 4 μM BAP in MS medium was combined with 1 μM NAA. However, 2 μM BAP in combination with 1 μM GA<sub>3</sub> resulted in 70% a lower shoot induction frequency, with only a

70% effectiveness with 1 μM GA<sub>3</sub> (Fig. 2b). These results are consistent with similar findings mature cotyledons of soybean, were 68% shooting frequency was obtained on 4.54 μM BAP while 50% response was obtained on 13.3 μM GA<sub>3</sub> (Franklin et al., 2004).

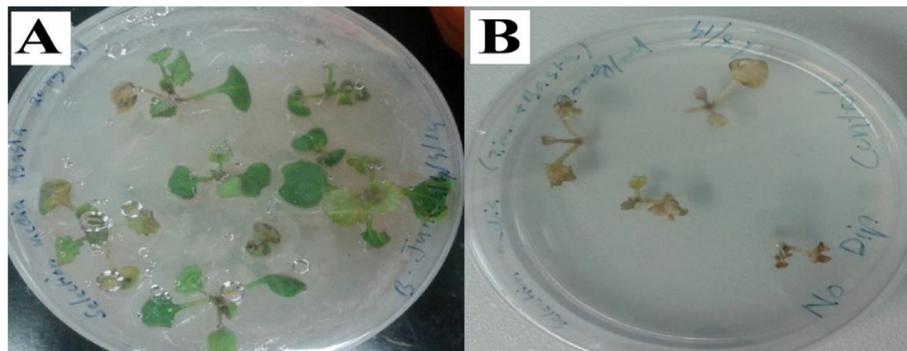
When considering the number of shoots per callus, 4 μM BAP produced the most shoots (11.1 ± 0.21); however, further increases in BAP concentration beyond 4 μM resulted in a drastic decline in the number of shoots per callus and eventually resulted in shoot inhibition at 10 μM (Fig. 3b and c). A significant increase in the number of shoots (17.3 ± 0.43) occurred with 4 μM BAP in combination with 1 μM NAA (Fig. 3a). An optimum shoot length of 5.7 cm was observed at 4 μM BAP which was further enhanced by addition of 1 μM NAA into the media containing 4 μM BAP (Fig. 4a). The role of BAP during *in vitro* morphogenesis is associated with metabolism of endogenous growth regulators i.e. cytokinins, auxins, GA<sub>3</sub> and ethylene (Radke-Yarrow et al., 1998). It is suggested that most probably, BAP enhances the susceptibility of plant tissues for exogenous and endogenous factors that are involved in differentiation and dedifferentiation of the plant cells *in vitro* (Khan et al., 2015). However, the choice of explants also plays a vital role in shoot organogenesis. Nonetheless, cotyledon explants are more potent than hypocotyl explants for callus mediated shoot organogenesis in *B. juncea* (Bano et al., 2010).

### 3.3. Root induction

Elongated shoots were then transferred into rooting media fortified with different levels of IAA, either alone or in combination with Kin. The elongated shoots initiated root formation at 6 μM of IAA. The higher concentration of IAA showed inhibitory effects on root organogenesis (Table 2). The rooting frequency was further



**Fig. 3.** Impact of plant growth regulator (BAP GA<sub>3</sub> and NAA) on shoot number in *B. juncea*. Values are mean of five replicates with ±SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are not significantly different at  $p \leq 0.5$ .



**Fig. 4.** Impacts of different PGRs on mean shoot length in *B. juncea*. Values are mean of five replicates with ± SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are significantly different at  $p \leq 0.5$ .

**Table 2**Impacts of different concentrations of IAA and its combinations with Kin on root growth parameters in *B. juncea*.

Treatments	Rooting (%)	No. of roots/shoot	Root-length (cm)
IAA (2 $\mu$ M)	32 $\pm$ 2.43	1.9 $\pm$ 0.43	3.8 $\pm$ 0.93
IAA (4 $\mu$ M)	47 $\pm$ 2.55	2.1 $\pm$ 0.73	4.2 $\pm$ 1.12
IAA (6 $\mu$ M)	58 $\pm$ 1.93	3.1 $\pm$ 0.83	6.2 $\pm$ 1.23
IAA (8 $\mu$ M)	42 $\pm$ 1.12	2.7 $\pm$ 0.43	4.4 $\pm$ 1.28
IAA (10 $\mu$ M)	38 $\pm$ 1.85	0.9 $\pm$ 0.23	3.1 $\pm$ 0.02
IAA (2 $\mu$ M) + Kin (1 $\mu$ M)	55 $\pm$ 1.93	2.7 $\pm$ 0.63	3.1 $\pm$ 0.23
IAA (4 $\mu$ M) + Kin (1 $\mu$ M)	65 $\pm$ 2.13	3.8 $\pm$ 1.93	5.2 $\pm$ 2.13
IAA (6 $\mu$ M) + Kin (1 $\mu$ M)	82 $\pm$ 3.43	5 $\pm$ 1.93	8.2 $\pm$ 1.23
IAA (8 $\mu$ M) + Kin (1 $\mu$ M)	75 $\pm$ 2.89	4.8 $\pm$ 1.33	5.5 $\pm$ 0.63
IAA (10 $\mu$ M) + Kin (1 $\mu$ M)	68 $\pm$ 2.33	3.5 $\pm$ 0.83	3.2 $\pm$ 0.13

Note: Values are mean of five replicates and observations were recorded after 4 weeks of culture.

enhanced when the elongated shoots were incubated in the medium supplemented with 6  $\mu$ M IAA in combination with 1  $\mu$ M Kn. With this particular PGR treatment, the highest rooting frequency (82%) with the maximum number of roots (5 roots/shoot) and the highest root length (8.2 cm) were observed (Table 2). Auxins, notably IAA and NAA, have been indicated as effective PGRs for rooting in different *Brassica* spp including *B. juncea* (Cogbill et al., 2010; Khan et al., 2009; Teo et al., 1997).

### 3.4. *Agrobacterium*-mediated *B. juncea* transformation and regeneration

A total of 200 *B. juncea* cotyledons were infected with *Agrobacterium* transformed with a binary transformation vector containing *EaDAcT*. Compared to other types of media, the highest regeneration frequency (100%) was observed on co-cultivation medium (Table 3). The incubation of explants for three days on co-cultivation medium was followed by transferring to selection medium 1. After 10–15 days on selection medium 1, creamy greenish callus formation at the cut ends was observed which eventually resulted in shoot formation. In our study, the responding frequency

**Table 3**

Growth % of transgenic plants with different medium.

Explants	PCM	SM-I	SM-II	PRM	TE
200	100	50	25	14	07

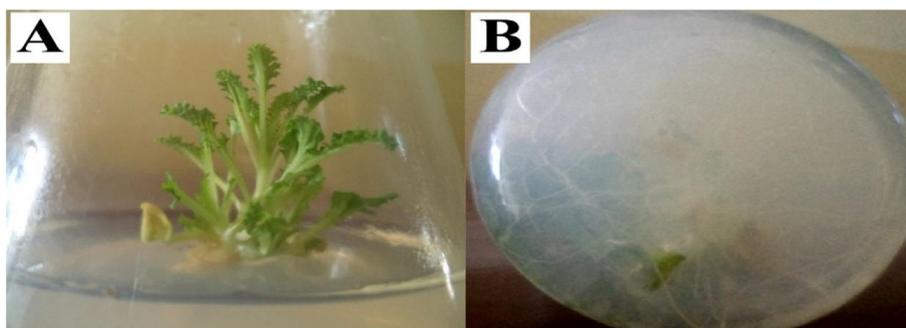
PCM: Plant Cultivation Medium, SM: Selection Medium PRM: Plant on rooting medium, TE: Transgenic efficiency.

of cotyledon explants was much higher for callus organogenesis than the previously published data on the exploitation of the same explant for callus formation followed by *Agrobacterium*-mediated transformation in *B. juncea* (Barfield and Pua, 1991; Das and Joshi, 2011; Dutta et al., 2005). Non-transgenic explants became yellow in color and did not respond *in vitro* on selection media 2 containing 25 mg l<sup>-1</sup> Basta and 25 mg l<sup>-1</sup> timintin. This media was found inhibitory for the growth of non-transgenic plants. Our study further suggests that half-cut cotyledons without meristem are better explants when used for transformation and regeneration because meristematic cells in the hypocotyl explants are often recalcitrant to *Agrobacterium* infection (De Block et al., 1989). The explants that were co-cultivated for more than 72 h period on co-cultivation medium could cause necrosis of the explants due to the excessive growth of Bacteria (Khan et al., 2003). After three weeks' culture period the transgenic explants were refreshed on selection medium 2, containing a higher level of basta (25 mg l<sup>-1</sup>) and multiple shoots were regenerated from the callus. The appropriate temperature contributes an important role in tissue culture transformation because it induces the expression of VIR genes, responsible for the transfer of T-DNA to the plant cells (Longdou et al., 2005). Therefore, for effective transformation, the growth chamber temperature was adjusted to 25 °C as at this temperature the *Agrobacterium* may efficiently transform the T-DNA region.

For rooting single shoots were excised and transferred to the rooting medium. Initially, small creamy callus was observed at the cut ends of the shoots followed by root organogenesis. After four weeks' culture period, well-established roots were formed (Fig. 5D and E).

### 3.5. Acclimatization and hardening

Rooted shoots were then shifted to plastic pots containing soil, vermiculite and horticulture along with osmocot (4:2:1) and the pots were covered with transparent cover bags and placed in acclimatization room at 21  $\pm$  1 °C with 70–90% humidity. The temperature was gradually increased up to 25 °C. The transparent covers were removed after three weeks for proper hardening. The acclimatization of plantlets by the gradual increase in temperature and use of polythene bags to control humidity improved the survival rate and hardening process (up to 78%) (Fig. 6). Numerous studies have reported vitrification in many *Brassica* species that is a variety-dependent process and can potentially cause problems in tissue culture transformation due to shoot maladjustment in the soil (Radke et al., 1988). Vitrification may be potentially reduced by using 0.5% agar in the rooting medium with perforation to assist with the release of CO<sub>2</sub> and ethylene gas easily.



**Fig. 5.** *In vitro* regeneration in *B. juncea*. (A) Callus formation from cotyledon explants after three weeks of culture (bar = 1.5 mm). (B) Shoot organogenesis in terms of mean shoot number (bar = 2.5 mm). (C) Shoot organogenesis in terms of mean shoot length (bar = 1 mm). (D, E) Root organogenesis (bar = 200  $\mu$ m).

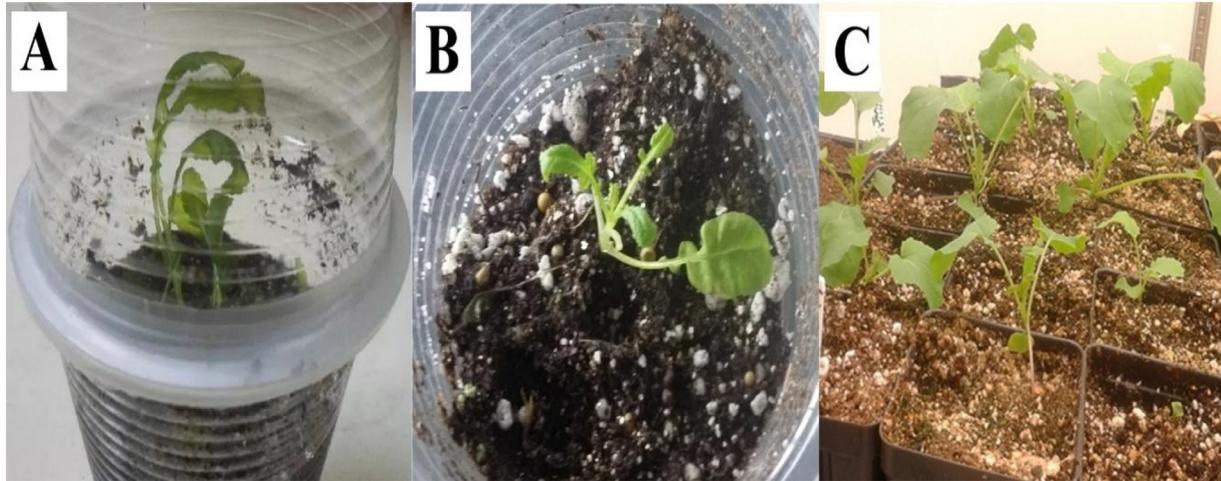


Fig. 6. Plants grown in different conditions. (A) Plant grown in covered pot. (B) Plant grown in open pot (C) plant grown in open field.

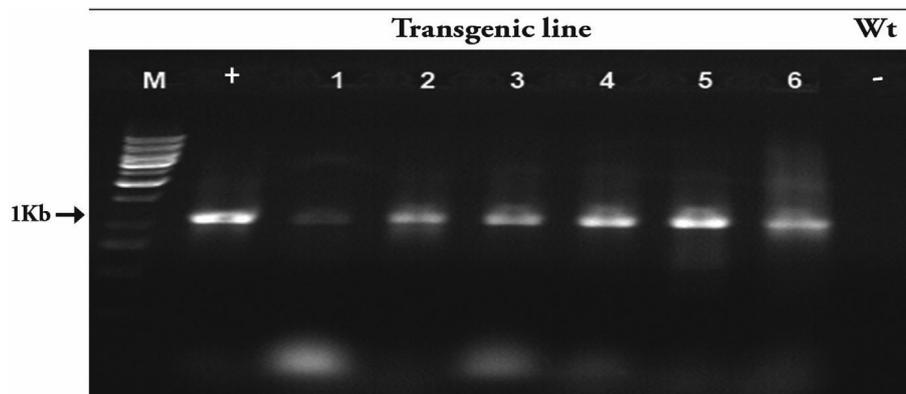


Fig. 7. Transformed *E. coli* culture overnight growth on solid LB medium in the presence of Kanamycin at 37 °C.

### 3.6. Molecular analysis of transgenic plants

PCR analysis of genomic DNA extracted from transgenic plants and control, was carried out by using specific primers for the *EaDAcT* gene (table 1). Here, the wild type plants did not show any amplification. For the confirmation of transformation, Transformed *E. coli* culture overnight growth on solid LB medium in the presence of Kanamycin at 37 °C (Fig. 7) and Transformation of the *B. juncea* plants was by the successful amplification of about 1 kb amplicon region of the *EaDAcT* gene as depicted in the positive control lane (Fig. 8). The amplification of the genomic DNA with *EaDAcT* primer confirmed the successful integration and the final transformation efficiency was found to be 7%. Moreover, for plant selection marker bar gene was used. The bar gene codes for the enzyme PAT, which activates the herbicide phosphinotricin (glufosinate) by acetylating it (Hara et al., 1988). This enzyme is a glutamate analog that inhibits glutamine synthetase thus accumulates more  $\text{NH}_4^+$  which is toxic for the growth of plant cells.

Moreover, *B. juncea* should be further screened out for its taxonomic, pharmacological, phytochemical, antimicrobial and genetic improvement to tolerate drought and salinity stress. As taxonomic studies like pollen (Ashfaq et al., 2018; Ayaz et al., 2019; Bahadur et al., 2018b, 2019a, 2019b; Gul et al., 2019c; Sufyan et al., 2018; Naz et al., 2019; Ullah et al., 2019a, 2019b) and foliar epidermal (Gul et al., 2019a, 2019b) have been found significant in the correct identification of species. Similarly, pharmacological (Rubab et al., 2019) and antimicrobial studies (Arif et al., 2018; Feroze et al.,

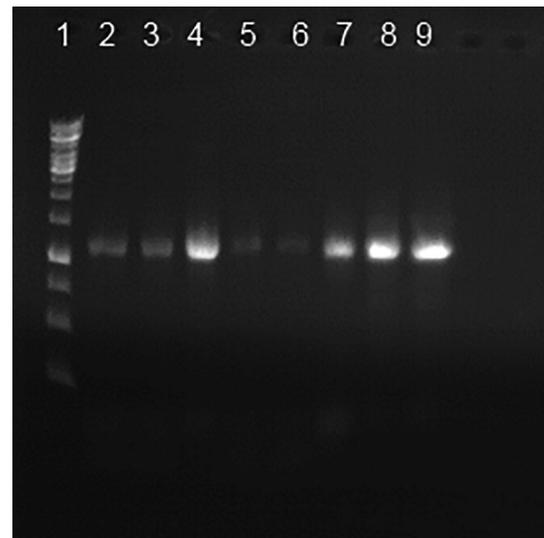


Fig. 8. PCR amplification of *EaDAcT* gene. Confirmation of transformants. Lane 2 is positive control (amplified from DNA construct) and 10, 11 represent negative controls. Lane 1 is 1 kb ladder (Quick-load 1 kb DNA ladder, NEB) and lanes 3–9 indicate transformed colonies. The size of construct is 1092 bp.

2019; Saqib et al., 2019a, 2019b; Sarah et al., 2019; Qasim Nasar et al., 2019) and genetic improvement (Kumar et al., 2018, 2014) have been shown the importance of medicinal plant).

#### 4. Conclusion

The present study comes to the conclusion with the results in which, highest callus formation frequency (87%) was observed with 4.0  $\mu\text{M}$  BAP. For shoot organogenesis, maximum shooting was observed in medium supplemented with 4.0  $\mu\text{M}$  BAP and 1.0  $\mu\text{M}$  NAA (92). However, at the concentration of 4  $\mu\text{M}$  BAP + 1  $\mu\text{M}$  NAA maximum shoots per explant (19 shoots/explant) was observed. The highest shoot length (6 cm) was recorded at BAP4  $\mu\text{M}$  plus NAA 1.0  $\mu\text{M}$ . For rooting IAA (6.0  $\mu\text{M}$ ) in combination with Kin (1.0  $\mu\text{M}$ ) was found best. The root induction frequency was 82%, with an average root length of 8.2 cm and mean number of 5 roots/shoot. The transformation efficacy was up to 7.0% using tissue culture-based transformation method.

#### Author's contributions

IN participated in the design of the study, carried out the experiments and drafted the manuscript. IM, TPD, AI, KA, MS, SB, KB, IK and MAA involved in the practical work and sample collection. SB, HK, IA, MS, AAS and FH, MS helped in manuscript writing and IM supervised the overall study. All authors read and approved the final manuscript.

#### Compliance with ethical standards

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#### The ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

#### Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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