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SCREENING OF PROMISING *BRASSICA NAPUS* L. GENOTYPES FOR CALLUS INDUCTION AND REGENERATION

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ABSTRACT

Ten different genotypes of *Brassica napus* L. were screened for callus induction and regeneration capabilities. The effect of different growth regulators and carbon sources on callus induction and regeneration was investigated using seven different callus induction media (CIM) compositions (CIM-1 to CIM-7) and five different shoot induction media (SIM) compositions (SIM-1 to SIM-5) along with controls. Among the callus induction media compositions supplemented with 3% sucrose or glucose as a carbon source, CIM-7 (Murashige and Skoog (MS) media supplemented with 4 mgL^{-1} NAA, 0.4 mgL^{-1} BAP and 0.4 mg L^{-1} Kinetin) resulted in maximum callus induction i.e. 80% and 69%, respectively. Among different SIM media supplemented with 3% sucrose or glucose, SIM-1(MS media supplemented with 0.3 mgL^{-1} NAA and 3 mg L^{-1} BAP) gave maximum shoot induction i.e. 16% and 9%, respectively. On shoot induction Driver-Kuniyuki walnut (DKW) media with 3% sucrose, maximum shoot induction (10%) was observed on SIM-1 (DKW media supplemented with 0.3 mgL^{-1} NAA and 3 mg L^{-1} BAP). Among the ten genotypes tested, the advanced line NIFA Nr.2 resulted in maximum callus induction (76%) on CIM media with 3% sucrose, whereas, the advanced line NIFA Nr.6 gave maximum callus induction (67%) on CIM containing 3% glucose. Maximum shooting (12.963%) on DKW media was observed for NIFA Nr.1. On SIM with 3% sucrose and glucose, the same line showed maximum shooting i.e. 18% and 10%, respectively. The shoots obtained were then transferred to half strength MS media with or without growth regulators (control media) for root development. The well rooted plants were transferred to soil and successfully grown under green house. The above mentioned combinations and concentrations provide base line information for future tissue culture based Brassica improvement programs especially via transgenic interventions.

Key words: *Brassica napus*, growth regulators, carbon sources, callus induction, regeneration

INTRODUCTION

Brassica napus L. is economically an important crop in Pakistan serving as one of the most important source of oil. *B. napus* is major target for improvement by the researchers and plant scientists via various approaches including breeding, tissue culture and molecular techniques due to its potential (Khan *et al*., 2013). The availability of an efficient *in vitro* system for regeneration is a prerequisite for tissues culture based improvement methods such as genetic transformation. However, efforts in this direction in Pakistan have generally met with limited success due to the recalcitrant nature of the Pakistani commercial canola cultivars. So there is pressing need to thoroughly evaluate the tissue culture responses of promising local *B. napus* genotypes. The initial step in plant tissue culture is callus induction. Development of callus in plants generally goes through three basic stages. During the first stage, induction of cell division occurs that is followed by de-differentiation, while in the last step either reduction in cell division occurs or cell division completely stopovers and within the callus, differentiation increases (George *et al.*, 2008). A variety of callus may be produced from a single ex-plant depending on the features like color, appearance and the degree of compaction and morphogenetic potential (George et al., 2008). Different biotic and abiotic factors affect the callus induction, growth and quality of callus for regeneration. These factors include type of ex-plant, species, cultivars, plant growth regulators, carbon sources, temperature and light (George *et al.*, 2008).

After the induction of callus, the second step is plant regeneration. In *Brassica,* regeneration is significantly variable and genotype specific (Dunwell, 1981). The existing information indicates that the regeneration through organogenesis has been accomplished using various tissues including cotyledons (Hachey *et al*., 1991; Ono *et al*., 1994), hypocotyls (Khehra and Mathias, 1992; Phogat *et al*., 2000), peduncle (Eapen and George, 1997), leaves (Radke *et al*., 1988), thin cell layers of epidermis and sub epidermis (Klimaszewska and Keller, 2002) and protoplasts (Hu *et al*., 1999).

In callus induction different plant hormones such as auxins and cytokinins (CKs) are used. Typically all plant cells can yield both auxins and CKs, however adolescent shoot organs are the major sites for auxin (IAA) generation and root tips are major sites of CKs production in plants (Aloni *et al,* 2006). In plant tissue culture, auxins are widely utilized for callus initiation (Chawla, 2002; George *et al*., 2008; Park *et al*., 2010). The auxins usually used in callus initiation are 2, 4-dichlorophenoxyacetic acid (2,4-D), Indole-3-acidic acid (IAA), Naphthalene acidic acid

(NAA) and Indole-3-butyric acid (IBA). CKs are derivatives of Adenine and are needed for adventitious shoot formation and stimulating cell division (Chawla, 2002; George *et al*., 2008). Major CKs used in plant tissue culture are Benzyl adenine (BA), 6-benzylaminopurine (BAP), Zeatin, Thidiazuron (TDZ) and Kinetin (Alam *et al*., 2010). In addition to plant hormones, many other studies have emphasized on the importance of concise concentration of carbohydrates on the development of embryos, callus induction and regeneration abilities (Ilic Grubor *et al.*, 1998 a, b; Bogunia and Przywara, 2000). In most of the reports regarding tissue culture and genetic transformation of *Brassica* species, *B. napus* has not yet been completely explored. An efficient regeneration protocol for *B. napus* is needed to be established for its use in transformation experiments. The present study was an attempt to develop and standardize an efficient and high frequency regeneration system for different genotypes of *B. napus***.**

MATERIALS AND METHODS

Abbreviations: CIM- Callus Induction Media; SIM- Shoot Induction Media; RIM- Root Induction Media; MS- Murashige and Skoog (1962) medium; BA- Benzyladenine; 2,4-D- 2,4-dichlorophenoxyaceticacid; NAA- α-naphthaleneaceticacid.

Plant materials

Seeds of *B. napus* genotypes were obtained from Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan. Two varieties viz. Durr-e-NIFA and Abasyn-95 and eight advanced lines i.e. NIFA Nr.1 to Nr.8 were used in this study.

Media preparation for seed germination

Half strength Murashige and Skoog (MS) media with Gamborg's vitamins (Phytotech USA) were prepared in distilled water. The pH was adjusted to 5.7 ± 0.1 by adding HCl or NaOH. 0.6% (6 gL^{-1}) agar was added to solidify the media. The media was sterilized by autoclaving at 15 psi, 121°C for 15-20 minutes. After sterilization, the media was poured into the petri dishes and allowed to cool inside a laminar flow unit (LFU).

Sterilization of plant materials

Selected healthy seeds of all the ten genotypes were washed three times with tap water followed by washing with distilled water three times. Then surface sterilization was carried out in a sequential manner with 70% ethanol for 2 min followed by 6% Sodium hypochlorite and two drops of Tween-20 for 5 min and subsequently rinsing 4 times with sterile water inside a LFU. Then, the seeds were placed on sterile filter paper (Whatman No.1) and allowed to dry.

Seed germination

The sterilized seeds (15-20 seeds/plate) were placed on half strength MS media supplemented with Gamborg's vitamins and were kept in the dark at 25 ± 3ºC for 3 days, then the seeds were transferred to light in growth room with 16 hours photoperiod for 4 days.

Callus media preparation

MS culture media with Gamborg's vitamins was prepared in distilled water and supplemented with different concentrations of growth regulators (Table 1). This media was used for callus induction. A carbon source (sucrose or glucose) was added in the media (30 gL^{-1}). The media was also supplemented with 0.5 gL^{-1} polyvinylpyrrolidone (PVP) and 0.5 gL^{-1} 2-(*N*-morpholino) ethane sulfonic acid (MES). The pH of the media was kept at 5.7 ± 0.1 using HCl or NaOH. To solidify the media, 0.6% agar was added (6 gL^{-1}). The media was autoclaved at 15 psi, 121ºC for 15-20 min, poured in petri dishes and allowed to cool.

Explant and their source

After germination for 7 days, hypocotyls of the *in vitro* grown plants were used as explants. The hypocotyls of uniform sizes (1 cm) were used to produce callus.

Initiation of callus

The experiments were performed in petri plates using 10-15 ml media per petri plate. Explants were removed from *in vitro* grown seedlings and cultured in the petri dishes. Each treatment was repeated three times. Callus initiation and production was observed regularly. Data on callus induction was recorded using the following formula:

$$\text{Callus Induction Frequencies (CIF)} = \frac{\text{Number of calli-producing explants}}{\text{Total number of explants in the culture}} \times 100$$

Shoot initiation media

Two types of shoot initiation media viz., MS media with Gamborg's vitamins and Driver and Kuniyuki (DKW) media were used for shoot initiation.

MS shoot initiation media preparation

MS culture media with Gamborg's vitamins was prepared in distilled water and supplemented with different concentrations of growth regulators (Table 4). The media was also supplemented with $AgNO_3$ (15 µM). This media was used for shoot induction. As a carbon source, 30 gL^{-1} sucrose or glucose (depending on experiment) was also added in the media. The pH of the media was kept at 5.7 ± 0.1 using HCl or NaOH. To solidify the media, 0.6% agar was added to the media. The media was autoclaved at 15 psi, 121ºC for 15-20 min, poured in flasks or bottles and allowed to cool.

DKW shoot initiation media preparation

DKW Basal media was prepared in distilled water and supplemented with Myo-Inositol 100 mgL^{-1}, Thiamine hydrochloride 2.00 mgL^{-1}, Nicotinic acid (Free acid) 1.00 mgL^{-1}, Glycine (Free base) 2.00 mgL^{-1} and different concentrations of growth regulators (Table 4). In addition the media was also supplemented with $AgNO_3$ (15 µM) because it plays an important role in efficient shoot regeneration by inhibiting ethylene action which is involved in recalcitrant *in vitro* shoot differentiation. This media was used for shoots regeneration. 30 gL^{-1} carbon source (sucrose or glucose) was used in preparation of the media. The pH of the media was kept at 5.7 ± 0.1 using HCl or NaOH. To solidify the media, 0.6% agar was added to the media.

Shoot initiation

The calli were transferred to shoot initiation media supplemented with different concentrations of growth regulators. The plant regeneration was performed in flasks or bottles. Each treatment was replicated three times. Shoot initiation was observed regularly. Data on shoot initiation was recorded using the following formula:

$$\text{Shoot Induction Frequencies (SIF)} = \frac{\text{Number of shoot-producing calli}}{\text{Total number of calli in the culture}} \times 100$$

Root initiation media preparation

Root initiation media was prepared in distilled water. Different concentrations of growth regulators (Appendix 1) were added in to the media. Additionally 30 gL^{-1} sucrose was added as carbon source. The pH of the media was kept at 5.7 ± 0.1 using HCl or NaOH. To solidify the media, 0.6% agar was added to the media.

Root initiation

The regenerated shoots were transferred to ½ MS media supplemented with different concentrations of growth regulators. The data on root initiation was observed regularly. The data on root initiation was calculated as

$$\text{Root Induction Frequencies (RIF)} = \frac{\text{Number of root-producing shoots}}{\text{Total number of shoots in the culture}} \times 100$$

Growth conditions

The temperature range for growth was 25 ± 3ºC in growth chamber, while a photo period of about 16 hours light and 8 hours dark was maintained.

Acclimatization and transferring to green house

The plantlets showing a well-developed root system were transferred to peat mosses, kept in the growth room at 25ºC under a 16/8 hours light/dark cycle for 15 days. After 15 days the Plants were transferred to green house and allowed to grow on the peat mosses for another 15 days. Then the plants were transferred to soil for acclimatization.

Data analysis

The data were analyzed statistically by using statistical package Statistix 8.1. In case of significant differences ($P \leq 0.05$), means were separated by LSD test.

RESULTS

Callus induction responses on different callus induction media in the presence of sucrose

Hypocotyls of the seven days old seedlings of *B. napus* genotypes were used as explants and transferred to different CIM supplemented with different combinations and concentrations of growth regulators using 3% sucrose as a carbon source (Table 1). Significant differences (P = 0.0000) were also observed among the ten genotypes used (Table 2). The average callus induction responses of the two genotypes i.e. advanced lines NIFA Nr. 5 and cultivar Durr-e-NIFA were poor (Ca. 26%) among all tested genotypes on all tested CIM compositions (Table 2). Based on overall mean performance on all tested CIM compositions, the advanced lines NIFA Nr. 2, NIFA Nr. 6 were found to be the most promising lines in terms of calli induction showing 77% and 74% callus induction frequencies, respectively. Among the media compositions used, significant differences (P = 0.0000) were observed. CIM-7 (MS media supplemented with 4 mgL^{-1} NAA, 0.4 mgL^{-1} Kinetin and 0.4 mgL^{-1} BAP) was found to be the most responsive medium with an average callus induction of 80% followed by CIM-6 (72%) while no callus induction was observed on control medium (Table 2). It is pertinent to note that even the two most recalcitrant genotypes i.e. advanced lines NIFA Nr. 5 and cultivar Durr-e-NIFA showed fairly good calli induction responses of 62% and 85%, respectively on CIM-7. Significant differences (P = 0.0000) were also seen in case of interactions. The highest callus induction (99.000%) was observed in Line NIFA Nr.6 on CIM-6 followed by NIFA Nr.2 (95.000%) on the same media while variety Durr-e-NIFA produced the lowest callus induction (8.333%) on CIM-4 (Fig. 1 and Table 2). Line NIFA Nr.2 and Nr.6 possess more callus induction capabilities compared to other genotypes.

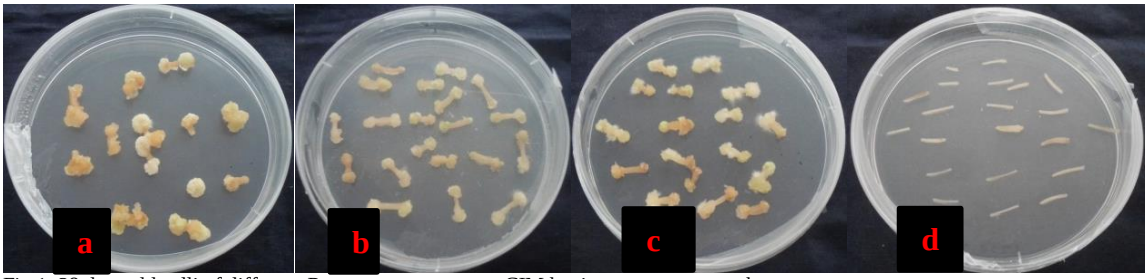

Fig 1. 28 days old calli of different *B. napus* genotypes on CIM having sucrose as a carbon source.
a) Calli produced by NIFA Nr. 6 on CIM-6 b) Calli produced by NIFA Nr. 2 on CIM-6 c) Calli produced by NIFA Nr. 6 on CIM-7 d) Calli produced by NIFA Nr. 6 on Control Media.

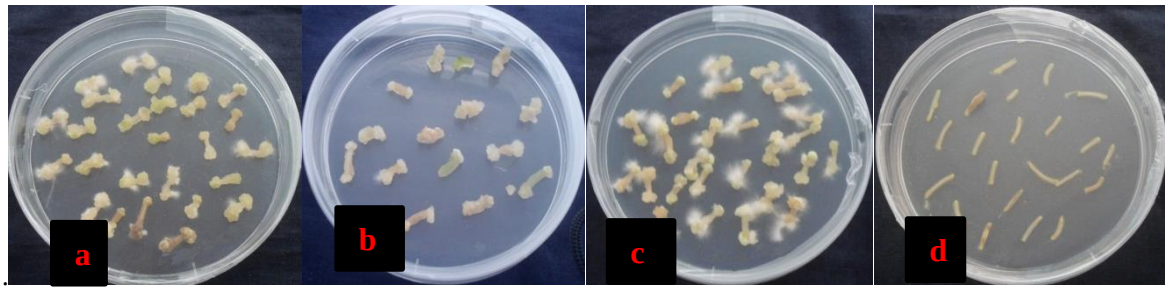

Fig. 2. 28 days old calli of different *B. napus* genotypes on CIM having glucose as a carbon source.
a) Calli produced by NIFA Nr. 6 on CIM-6 b) Calli produced by NIFA Nr. 2 on CIM-6 c) Calli produced by NIFA Nr. 6 on CIM-7 d) Calli produced by NIFA Nr. 6 on Control Media.

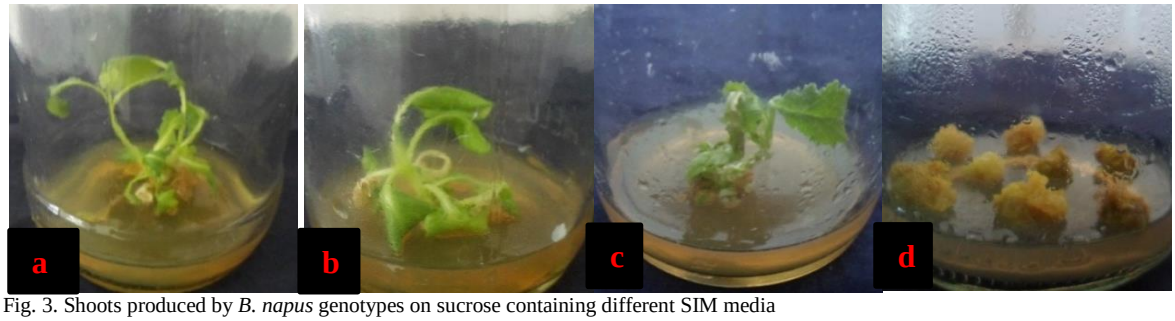

Fig. 3. Shoots produced by *B. napus* genotypes on sucrose containing different SIM media
a) Shoots produced by NIFA Nr. 1 on SIM-1 b) Shoots produced by NIFA Nr. 1 on SIM-4
c) Shoots produced by NIFA Nr. 6 on SIM-1 d) NIFA Nr. 1 on control media.

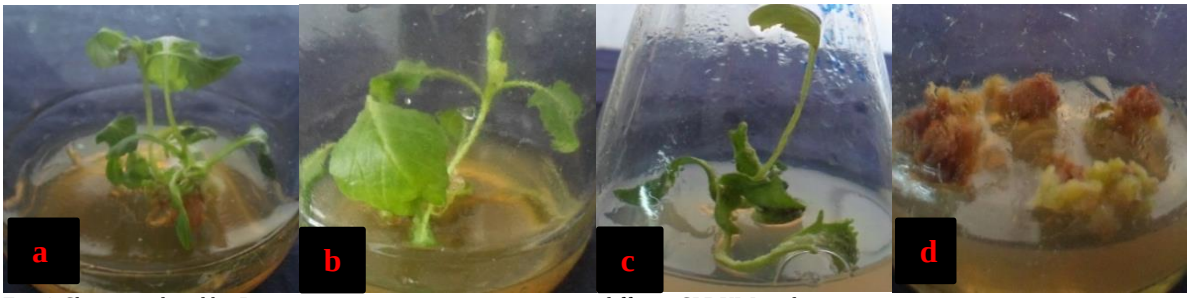

Fig. 4. Shoots produced by *B. napus* genotypes on sucrose containing different SI DKW media
a) Shoots produced by NIFA Nr. 1 on SIM-1 b) Shoots produced by NIFA Nr. 6 on SIM-1
c) Shoots produced by NIFA Nr. 1 on SIM-4 d) NIFA Nr. 1 on control media.

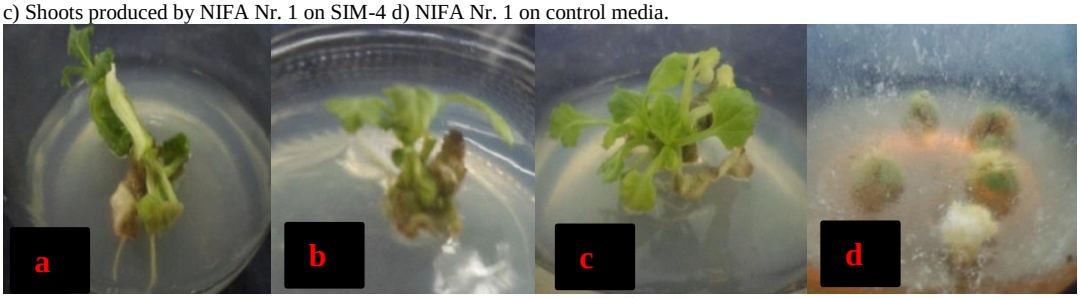

Fig. 5. Shoots produced by *B. napus* genotypes on glucose containing different SIM media
a) Shoots produced by NIFA Nr. 1 on SIM-1 b) Shoots produced by NIFA Nr. 6 on SIM-1
c) Shoots produced by NIFA Nr. 1 on SIM-4 d) NIFA Nr. 1 on control media.

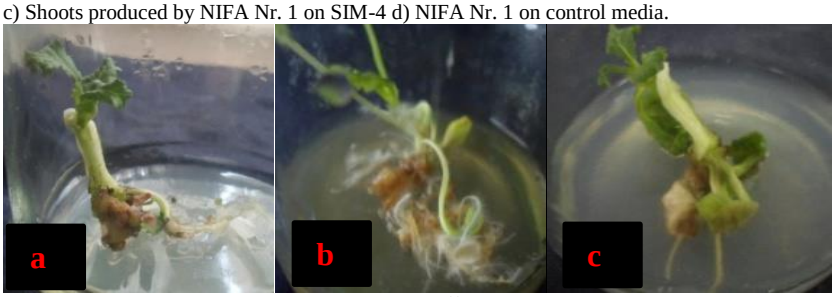

Fig. 6. Roots produced by *B. napus* genotypes on different root induction media (RIM).
a) Roots produced by NIFA Nr. 1 on control media b) Roots produced by NIFA Nr. 6 on RIM-1 c) Roots produced by NIFA Nr. 1 on RIM-2.

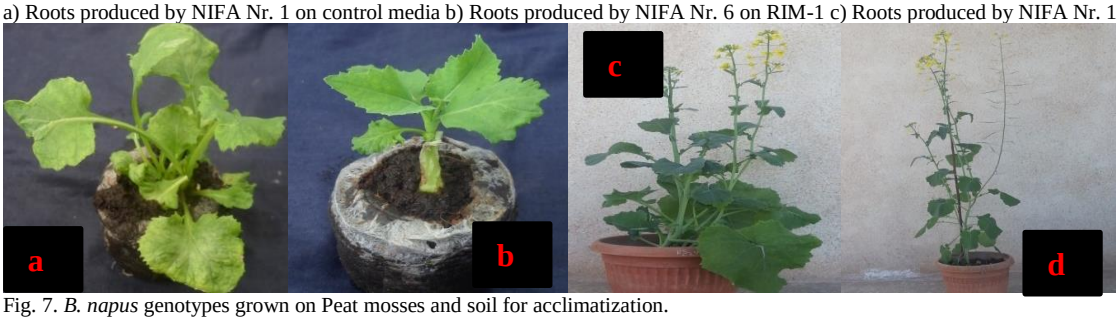

Fig. 7. *B. napus* genotypes grown on Peat mosses and soil for acclimatization.
a and b both shows NIFA Nr.1 plants grown on peat mosses in green house at IBGE
c and d both shows NIFA Nr.1 plants grown on soil in green house at IBGE.

Callus induction responses on different callus induction media in the presence of glucose

After seven days of germination, the hypocotyls were cut into small sections of about 1 cm. These pieces were transferred to control media and different CIM formulations augmented with different concentrations of growth regulators in the presence of 3% glucose. The evaluated genotypes exhibited significant differences (P = 0.0000) to callus induction (Table 3).

Just like the different CIM formulations augmented with 3% sucrose, the average callus induction responses of the same two genotypes i.e. advanced lines NIFA Nr. 5 and cultivar Durr-e-NIFA were again poor i.e. 26% and 20%, respectively on all tested CIM compositions in the presence of glucose (Table 3). Based on overall mean performance on all tested CIM compositions, albeit lesser calli induction responses as judged from the lower mean values compared to sucrose augmented CIM, the evaluated genotypes in general exhibited a similar pattern. In this

context, the advanced lines NIFA Nr. 6, NIFA Nr. 8, NIFA Nr. 2, were found to be the most promising lines in terms of calli induction showing 67%, 63% and 62% calli induction responses, respectively. Regarding the individual performance of different genotypes on different CIM in the presence of glucose, again these two advanced lines i.e. NIFA Nr. 2, NIFA Nr. 6 were found to be superior among all tested genotypes as judged from the individual calli induction responses of 95% and 99% , respectively on CIM-6.

Among the seven media compositions used, significant differences (P = 0.0000) were observed. CIM-7 was again found to be the best media with the highest callus induction (69%) followed by CIM-5 (64%), while control medium gave no callus induction (Table 3). It is pertinent to note that in spite of smaller values for calli induction responses compared to CIM-7 augmented with sucrose, CIM-7 can be regarded as the most suitable general CIM formulation for all genotypes which is evident from the fact that even the two most recalcitrant genotypes i.e. advanced lines NIFA Nr. 5 & cultivar Durr-e-NIFA showed fairly good calli induction responses of 52% and 71%, respectively on CIM-7.

In case of interactions significant differences (P = 0.0000) were also detected. Line NIFA Nr.6 gave the highest callus induction (92%) on CIM-6 followed by NIFA Nr.2 (86%) on the same media while variety Durr-e-NIFA gave minimum callus induction (8%) on CIM-4 (Fig. 2 and Table 3).

Regeneration responses on different shoot induction media in the presence of sucrose

Twenty eight days old calli of the ten different genotypes of *B. napus* were placed on different shoot induction media (SIM) supplemented with different concentration of growth regulators and control media for shoots induction (Table 4) in the presence of sucrose. Significant differences (P = 0.0000) were noticed among the media compositions and the genotypes used (Table 5). Among the five media formulations tested, SIM-1 (MS media supplemented with 0.3 mgL^{-1} NAA and 3 mgL^{-1} BAP) was the most responsive medium with an average shoot initiation of 16% followed by SIM-4 (13%), while control media gave no shoot induction (Table 5). Thus irrespective of the genotypes, these two media formulations i.e. SIM-1 and SIM-4 showed regeneration responses up to some extent in evaluated genotypes. These results showed that precise amount of growth regulators played a vital role in shoot induction. Similarly, among the ten genotypes evaluated, the highest shoot induction (18%) was observed in case of line NIFA Nr.1 followed by NIFA Nr.6 (15%) while variety Durr-e-NIFA produced minimum shoots (2%). Significant differences (P = 0.0000) were also detected in case of interactions. The highest shoot induction) was recorded for NIFA Nr.1 on SIM-1 (36%) and on SIM-4 (31%) followed by NIFA Nr.6 (31%) on SIM-1 (Fig. 3 and Table 5), while a number of genotypes failed to induce shoots on different media compositions. These results indicated that for shoot induction of a particular genotype the media should be supplemented with a right concentration and combination of growth regulators.

Table 1. Concentrations of Growth Regulators in Callus Induction Media (CIM).

CIM	Auxins ($mg.L^{-1}$)	Cytokinins ($mg.L^{-1}$)	
	2,4-D	BAP	Kinetin
CIM-1	1	0	1
CIM-2	2	0.2	0.2
CIM-3	3	0.3	0.3
CIM-4	4	0.4	0.4
	NAA	BAP	Kinetin
CIM-5	2	0.2	0.2
CIM-6	3	0.3	0.3
CIM-7	4	0.4	0.4
Control Media	0	0	0

Least Significant Difference (LSD) values for genotypes and media compositions calculated by using statistical package statistix 8.1 were 2.8939 and 2.5884 respectively, while for interactions between genotypes and media compositions was 8.1852. Means for each category followed by the same letters do not differ significantly from one another at 5% level of significance. The left column in the table shows CIM compositions while the right column shows mean percent value for callus induction on different CIM. The middle columns show percent callus induction values for different genotypes on different CIM. The row at the bottom of the table shows mean percent value of callus induction for different genotypes.

Table 2. Mean percent values of callus induction of *B. napus* genotypes on different CIM containing sucrose as a carbon source.

Media Compositions	Genotypes										Means
	Durr-e-NIFA	Abasyn-95	NIFA Nr.1	NIFA Nr.2	NIFA Nr.3	NIFA Nr.4	NIFA Nr.5	NIFA Nr.6	NIFA Nr.7	NIFA Nr.8	
CIM-1	28.33 X	28.33 X	62.33 STU	80.00 HIJKLM	85.00 FGHIJ	46.33 V	10.33 Z	70.66 NOPQR	56.33 U	79.00 IJKLM	54.66 E
CIM-2	25.00 X	45.66 V	66.66 PQRST	78.67 JKLMN	64.33 QRSTU	62.66 RSTU	46.66 V	64.33 QRSTU	60.00 TU	81.33 HIJKL	59.53 D
CIM-3	25.00 X	13.33 YZ	86.66 CDEFGHIJ	91.33 ABCDEFG	87.66 BCDEFGH	73.66 LMNOP	37.33 W	87.67 BCDEFGH	80.00 HIJKLM	73.00 MNOP	65.56 C
CIM-4	8.33 Z	66.66 PQRST	78.66 JKLMN	80.00 HIJKLM	83.33 GHIJK	72.33 MNOPQ	12.66 YZ	93.33 ABCDE	78.66 JKLM N	85.66 DEFG HIJ	65.96 C
CIM-5	20.00 Y	80.00 HIJKLM	78.66 JKLMN	94.33 ABC	89.67 BCDEFG	86.66 CDEFGHIJ	20.67 XY	83.33 GHIJK	83.33 GHIJK	68.33 OPQRS	70.50 B
CIM-6	13.00 YZ	87.00 BCDEFGHI	78.66 JKLMN	95.00 AB	68.67 OPQRS	93.60 ABCD	15.00 YZ	99.00 A	91.33 ABCDEFG	75.00 LMNO	71.66 B
CIM-7	85.33 EFGHIJ	90.00 BCDEFG	73.33 LMNOP	92.66 ABCDEF	75.65 KLMNO	66.60 PQRST	62.33 STU	94.66 ABC	73.00 MNOP	90.00 BCDEFG	80.36 A
Control Media	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00 F
Means	25.66 E	51.37 D	65.62 C	76.50 A	69.29 B	62.75 C	25.62 E	74.12 A	65.33 C	69.04 B	

Least Significant Difference (LSD) values for genotypes and media compositions calculated by using statistical package statistix 8.1 were 4.0612 and 3.6325 respectively, while for interactions between genotypes and media compositions was 11.487. Means for each category followed by the same letters do not differ significantly from one another at 5% level of significance. The left column in the table shows CIM compositions while the right column shows mean percent value for callus induction on different CIM. The middle columns show percent callus induction values for different genotypes on different CIM. The row at the bottom of the table shows mean percent value of callus induction for different genotypes.

Table 3. Mean percent values of callus induction of *B. napus* genotypes on different CIM containing glucose as a carbon source.

Media Composition	Genotypes										Means
	Durr-e-NIFA	Abasyn-95	NIFA Nr.1	NIFA Nr.2	NIFA Nr.3	NIFA Nr.4	NIFA Nr.5	NIFA Nr.6	NIFA Nr.7	NIFA Nr.8	
CIM-1	18.33 TU	20.00 T	68.33 FGHIJK LM	71.00 CDEFG HIJKL	77.66 BCDEF G	36.00 RS	16.00 TU	73.66 CDEF GHI	45.00 PQR	65.33 IJKLM N	49.13 D
CIM-2	16.66 TU	33.00 S	60.00 LMNO	68.66 FGHIJK LM	56.33 NOP	60.00 LMNO	20.00 T	58.00 MNO	50.66 OPQ	69.66 EFGHI JKL	49.30 D
CIM-3	18.33 TU	10.66 TUV	75.00 BCDEF GHI	60.33 LMNO	66.66 GHIJKL MN	66.66 GHIJK LMN	33.33 S	75.33 BCDE FGHI	66.00 HIJKL MN	77.00 BCDE FGH	54.93 C
CIM-4	8.00 UV	56.33 NOP	68.33 FGHIJK LM	68.66 FGHIJK LM	78.66 BCDEF	61.00 KLMN O	12.00 TU	80.33 BCDE	72.66 CDEF GHIJ	75.00 BCDE FGHI	58.10 C
CIM-5	15.33 TU	73.00 CDEFG HIJ	72.33 CDEFG HIJK	81.66 ABCD	43.33 QRS	75.66 BCDE FGHI	55.66 NOP	72.66 CDEF GHIJ	75.00 BCDE FGHI	74.00 CDEF GHI	63.86 B
CIM-6	11.00 TUV	76.00 BCDEF GHI	60.33 LMNO	86.00 AB	68.66 FGHIJK LM	77.66 BCDE FG	15.00 TU	92.00 A	78.00 BCDE FG	65.33 IJKLM N	63.00 B
CIM-7	70.66 DEFGHI JKL	75.33 BCDEF GHI	65.66 HIJKLM N	62.00 JKLMN O	70.33 DEFGHI JKL	67.00 GHIJK LMN	52.00 OPQ	82.33 ABC	65.66 HIJKL MN	76.66 BCDE FGHI	68.76 A
Control media	0.00V	0.00V	0.00V	0.00V	0.00V	0.000V	0.00V	0.00V	0.00V	0.00V	0.00E
Means	19.79 G	43.04 E	58.75 CD	62.29 BC	57.70 D	55.50 D	25.50 F	66.79 A	56.62 D	62.87 AB	

Table 4. Concentrations of Growth Regulators in Shoot Induction Media (SIM).

SIM	Auxins (mgL^{-1})	Cytokinins (mgL^{-1})
	NAA	BAP
Control Media	0	0
SIM-1	0.3	3
SIM-2	1	3
	NAA	Kinetin
SIM-3	1	3
	2,4-D	BAP
SIM-4	2	0.5
SIM-5	1	3

Mean percent values of shoot induction of *B. napus* genotypes on sucrose containing different shoot induction (SI) MS media. Least Significant Difference (LSD) values for genotypes and media compositions calculated by using statistical package statistix 8.1 were 2.7037 and 2.0943 respectively, while for interactions between genotypes and media compositions was 6.6227. Means for each category followed by the same letters do not differ significantly from one another at 5% level of significance. The left column in the table shows SIM compositions while the right column shows mean percent value for shoot induction on different SIM. The middle columns shows percent values for shoot induction of different genotypes on different SIM. The row at the bottom of the table shows mean percent value of shoot induction for different genotypes.

Table 5. Mean percentage value of shoot induction on different SIM media.

Media Composition	Genotypes										Means
	Durr-e-NIFA	Abasyn-95	NIFA Nr.1	NIFA Nr.2	NIFA Nr.3	NIFA Nr.4	NIFA Nr.5	NIFA Nr.6	NIFA Nr.7	NIFA Nr.8	
Control Media	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 H	0.00 E
SIM-1	6.67 FG	6.67 FG	35.53 A	6.66 FG	13.33 DE	6.67 FG	8.89 EF	31.11 AB	26.66 B	13.33 DE	15.55 A
SIM-2	0.00 H	0.00 H	26.66 B	0.00 H	0.00 H	0.00 H	0.00 H	15.55 CD	13.33 DE	2.22 GH	5.77 C
SIM-3	0.00 H	0.00 H	8.89 EF	0.00 H	4.44 FGH	0.00 H	0.00 H	6.66 FG	8.89 EF	0.00 H	2.89 D
SIM-4	4.44 FGH	6.66 FG	31.11 AB	6.66 FG	8.89 EF	6.66 FG	6.66 FG	26.66 B	20.00 C	8.89 EF	12.66 B
SIM-5	0.00 H	0.00 H	6.66 FG	0.00 H	4.44 FGH	0.00 H	0.00 H	6.66 FG	4.44 FGH	0.00 H	2.22 D
Means	1.85 D	2.22 D	18.14 A	2.22 D	5.18 C	2.22 D	2.59 CD	14.44 B	12.22 B	4.074 CD	

Least Significant Difference (LSD) values for genotypes and media compositions calculated by using statistical package statistix 8.1 were 2.5612 and 1.9839 respectively, while for interactions between genotypes and media compositions was 6.2735. Means for each category followed by the same letters do not differ significantly from one another at 5% level of significance. The left column in the table shows SIM compositions while the right column shows mean percent value for shoot induction on different SIM. The middle columns show percent shoot induction values for different genotypes on different SIM. The row at the bottom of the table shows mean percent value of shoot induction for different genotypes.

Regeneration responses on different shoot induction media in the presence of glucose

On SIM supplemented with glucose, significant differences (P = 0.0000) were observed among the media compositions and the genotypes used. Among the five media formulations tested, irrespective of the carbon source and genotypes, SIM-1 produced maximum shoots (9%) followed by SIM-4 (7%) while control media gave no shoots (Table 6). Similarly, among the ten genotypes assessed, the highest shoot induction (10%) was observed in case of line NIFA Nr.1 followed by NIFA Nr.6 (8%), while variety Durr-e-NIFA resulted in the lowest shoot induction (0.7%). Non-Significant differences (P=0.1692) were detected in case of interactions. The highest shoot induction

(20%) was recorded for NIFA Nr.1 on SIM-1 followed by NIFA Nr.6 (18%) on the same media and NIFA Nr.1 (18%) on SIM-4 (Fig. 4 and Table 6), while a number of genotypes failed to induce shoots on different media compositions.

Table 6. Mean percent values of shoot induction of *B. napus* genotypes on glucose containing different SI MS media.

Media Composition	Genotypes										Means
	Durr-e-NIFA	Abasyn-95	NIFA Nr.1	NIFA Nr.2	NIFA Nr.3	NIFA Nr.4	NIFA Nr.5	NIFA Nr.6	NIFA Nr.7	NIFA Nr.8	
Control Media	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 C
SIM-1	2.22	4.44	20.00	4.44	6.66	4.44	4.44	17.77	13.33	8.89	8.66 A
SIM-2	0.00	0.00	13.33	0.00	0.00	0.00	0.00	8.88	6.67	0.00	2.88 B
SIM-3	0.00	0.00	6.66	0.00	2.22	0.00	0.00	4.44	6.66	0.00	2.00 B
SIM-4	2.22	4.44	17.77	2.22	4.44	4.44	4.44	13.33	8.89	6.66	6.88 A
SIM-5	0.00	0.00	4.44	0.00	2.22	0.00	0.00	4.44	2.22	0.00	1.33 BC
Means	0.74 C	1.48 C	10.36 A	1.11 C	2.59 C	1.48 C	1.48 C	8.14 AB	6.30 B	2.59 C	

Least Significant Difference (LSD) values for genotypes and media compositions calculated by using statistical package statistix 8.1 were 2.5189 and 1.9511 respectively, while for interactions between genotypes and media compositions was 6.1700. Means for each category followed by the same letters do not differ significantly from one another at 5% level of significance. The left column in the table shows SIM compositions while the right column shows mean percent value for shoot induction on different SIM. The middle columns show percent shoot induction values for different genotypes on different SIM. The row at the bottom of the table shows mean percent value of shoot induction for different genotypes.

Table 7. Mean percent values of shoot induction of *B. napus* genotypes on sucrose containing different SI DKW media.

Media Composition	Genotypes										Means
	Durr-e-NIFA	Abasyn-95	NIFA Nr.1	NIFA Nr.2	NIFA Nr.3	NIFA Nr.4	NIFA Nr.5	NIFA Nr.6	NIFA Nr.7	NIFA Nr.8	
Control Media	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 G	0.00 D
SIM-1	2.22 FG	2.22 FG	26.66 A	4.44 EFG	8.89 DE	4.44 EFG	6.67 EF	20.00 B	13.33 CD	6.66 EF	9.55 A
SIM-2	0.00 G	0.00 G	17.77 BC	0.00 G	0.00 G	0.00 G	0.00 G	13.33 CD	6.66 EF	0.00 G	3.77 C
SIM-3	0.00 G	0.00 G	6.67 EF	0.00 G	0.00 G	0.00 G	0.00 G	4.44 EFG	6.66 EF	0.00 G	1.77 D
SIM-4	2.22 FG	2.22 FG	20.00 B	2.22 FG	6.66 EF	4.44 EFG	4.44 EFG	13.33 CD	8.88 DE	4.44 EFG	6.88 B
SIM-5	0.00 G	0.00 G	6.66 EF	0.00 G	2.22 FG	0.00 G	0.00 G	4.44 EFG	4.44 EFG	0.00 G	1.77 D
Means	0.74 D	0.74 D	12.96 A	1.11 D	2.96 D	1.48 D	1.85 D	9.26 B	6.67 C	1.85 D	

Appendix 1. Concentrations of Growth Regulators in Root Induction Media (RIM).

RIM	Auxins (mgL^{-1})	Cytokinins (mgL^{-1})
	NAA	BAP
Control Media	0	0
RIM-1	0.5	0
RIM-2	0.3	3
	NAA	IBA
RIM-3	0	0.2
RIM-4	0	0.3
RIM-5	2	1
	IAA	BAP
RIM-6	0.1	0.2
	IAA	IBA
RIM-7	0.125	0.250

Regeneration responses on DKW media

Significant differences (P=0.0000) were observed among the media compositions and genotypes used. SIM-1 (DKW media supplemented with 0.3 mgL^{-1} NAA and 3 mgL^{-1} BAP) were found to be the best medium with 9.5563% shoot induction followed by SIM-4 (6.8890%), while control media contributed 0% shoot induction (Table 7). Among the ten genotypes evaluated, the highest shoot induction (12.963%) was noticed in line NIFA Nr.1 followed by NIFA Nr.6 (9.260%), while both varieties viz., Durr-e-NIFA and Abasyn-95 resulted in the lowest shoot induction (0.741%) (Table 7). Significant differences (P=0.0008) were also observed in case of interactions. The highest shoot induction (26.667%) was recorded in NIFA Nr.1 on SIM-1 followed by both lines NIFA Nr.6 on the same media and NIFA Nr.1 on SIM-4 (20.000% each) (Fig. 5 and Table 7), while a number of genotypes failed to induce shoots on different media compositions.

Root induction and acclimatization to green house conditions

Different media compositions were assessed for roots induction. Among these media compositions, roots were successfully induced on control media in NIFA Nr. 1 (Fig. 6a), RIM-1 in NIFA Nr. 6 (Fig. 6b) and RIM-2 in NIFA Nr.1 (Fig. 6c). The remaining genotypes including NIFA Nr.2, 3, 4, 5, 7, 8, Abasyn-95 and Durr-e-NIFA did not responded to root induction on the tested media compositions. The root induction frequencies were found very low. The well rooted shoots were transferred to jiffy pellets and kept in growth room for 15 days followed by transferring to green house for 15 days (Fig. 7a and b). The plants were then transferred to soil for acclimatization (Fig. 7c and d).

DISCUSSION

Tissue culture responses are highly genotype dependent

The present study was conducted with the aim to screen and select the most promising genotype(s) of *B. napus* for callus induction and subsequent regeneration and to figure out some sort of generalized formulations where diverse genotypes may respond to callus induction and regeneration. For this purpose ten different genotypes, including two varieties viz. Durr-e-NIFA and Abasyn-95 and eight advanced lines (NIFA Nr.1 to Nr.8) were evaluated. In our study, the callus induction response showed high rate of genotype dependency and varying response to the same media composition used for different genotypes. Among the ten genotypes evaluated, advanced line NIFA Nr. 2 and NIFA Nr. 6 were found best genotypes for callus induction. All other genotypes showed significant difference in terms of producing calli with the variety Durr-e-NIFA being the lower callus producing genotype. In line with our findings, varying calli induction responses for four different *B. napus* genotypes i.e. Dunkeld, Oscar, H-19 and Rainbow have been reported previously (Munir *et al.*, 2008). Similarly, Al-Naggar *et al*, (2010) evaluated five different varieties of *B. napus* (Serw 4, Serw 8, Pactol, Topus and Silvo) to determine the best genotype for callus induction. They observed that Serw 4 gave maximum calli formation compared to other genotypes; again corroborating our results that calli induction responses are highly genotype dependent. In case of regeneration, among the ten genotypes evaluated in the present study, line NIFA Nr.1 and NIFA Nr. 6 were again found the best genotypes for shoot and root induction. The remaining genotypes produced varying amount of shoots with the variety Durr-e-NIFA being the lower shoot producing genotype. The regeneration response showed high rate of genotype dependency and varying response to the same media composition used for different genotypes. In

this context our results are similar to the previous reports where five different varieties (Cyclone, Dunkled, Oscar, Rainbow and KS75) of *B. napus* were demonstrated to have varying regeneration responses (Khan *et al*., 2003). The differences in performance to calli induction and regeneration demonstrated that genetic makeup of the genotypes are key determinants for successful tissue culture responses.

Role of growth regulators in tissue culture responses

Growth regulators plays vital role in callus induction and regeneration. Among the different media combinations used in the present study, CIM-7 (MS media supplemented with 4 mg/L NAA, 0.4 mg/L BAP and 0.4 mg/L Kinetin) gave maximum callus induction. The results of this study showed that media supplemented with NAA gave good results in callus induction compared to media with 2,4-D. Significant variations in the formulation of growth regulators in terms of calli formation have been reported in numerous studies (Afshari *et al*., 2011; Alam *et al*., 2013; Munir *et al*., 2008) which along with other factors may partly be attributed to the differences in the genetic makeup of the genotypes used in the different studies. However, it is pertinent to note that irrespective of the genetic make of the ten different genotypes used in our studies, the compositions of growth regulators mentioned in CIM-7 media was good enough to induce callus induction responses in all genotypes. The significant callus induction responses induced by CIM-7 media formulation even in the two most recalcitrant genotypes i.e. advanced lines NIFA Nr. 5 (62%) & cultivar Durr-e-NIFA (85%), indicated that the combination and concentrations of growth regulators mentioned in CIM-7 can be taken as guide lines to figure out callus induction responses for unknown *B. napus* genotypes.

In case of shoot formation, SIM-1 (MS media supplemented with 0.3 mgL^{-1} NAA and 3 mgL^{-1} BAP) and SIM-4 (MS media supplemented with 2 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BAP) was found the best medium to induce shoots regeneration responses. A medium of similar composition was found stimulatory for shoot regeneration by Khan *et al.* (2002a). Alam *et al*. (2013) reported maximum shooting on media containing 3.0 mgL^{-1} BAP, 0.1 mgL^{-1} NAA and 5.0 mgL^{-1} $AgNO_3$. Although apart from genotype dependency, a number of factors may be contribute towards overall regeneration responses, but in the context of study it is noteworthy that irrespective of the genotypes, the two media formulations i.e. SIM-1 and SIM-4 showed regeneration responses up to some extent in all genotypes. These two formulations therefore can be taken as a starting point for the evaluation or further optimization of shoots regeneration responses in unknown genotypes in future studies.

Roots were successfully induced on control media, media supplemented with 0.5 mgL^{-1} NAA and on media supplemented with 0.3 mgL^{-1} NAA and 3 mgL^{-1} BAP. Burbulis *et al*. (2008) obtained shoots with well-developed roots on ½ strength MS media supplemented with 0.1 mgL^{-1} NAA. Alam *et al* (2013) reported media amended with 2.5 mgL^{-1} NAA as the best medium for root development. On the contrary, Khan *et al.* (2002b) observed maximum root induction in *B. napus* genotypes on MS media augmented with 0.250 mgL^{-1} IBA and 0.125 mgL^{-1} IAA.

Role of carbon sources in tissue culture responses

Tissue culture process is greatly influenced by the type and concentration of carbohydrates. They provide energy and help in the regulation of osmotic potential of the medium. Several studies have shown that carbohydrate also have role in signal transduction, gene expression and plant development (Zimmerman and Cobb, 1989). In the present study, effect of sucrose and glucose were evaluated on callus induction and regeneration. The results showed that sucrose resulted in higher callus induction and regeneration compared with glucose. Similar results were obtained by Cristea *et al*. (2012). They analyzed effect of sucrose, glucose, fructose and maltose on regeneration of *B. oleraceae.* They observed that sucrose resulted in maximum androgenic plant regeneration followed by maltose and glucose, while fructose was found less appropriate for regeneration. Slesak and Przywara (2003) found that embryos developed at higher rate in *B. napus* on medium supplemented with sucrose, followed by maltose and glucose. Fructose did not stimulate embryo growth. They also observed that frequency of developing embryos increased with sugar concentration, but normal embryogenesis occurred only on 1% sucrose and maltose. On higher concentration callus or shoots were observed. On media with 6% sucrose and 12% maltose, shoots and somatic embryos were produced. Baskaran and Jayabalan (2005) reported higher shoot regeneration frequency on media supplemented with sucrose compared to glucose and fructose for *Eclipta alba.* The results of Amutha *et al.* (2003) were found parallel to our results. They reported sucrose as better carbon source for inducing maximum regeneration compared to glucose, fructose and maltose. In contrast, Salvi *et al*. (2002) reported glucose as best carbon source for *in vitro* multiplication of turmeric cv. 'elite'. They observed significant increase in shoot and root length and also in number of roots on media supplemented with glucose. According to Cuenca and Vieitez (2000) the most effective carbon source for auxiliary branching and adventitious shoots regeneration under *in vitro* conditions was glucose.

Our results revealed that MS media responded better to shoot induction compared to DKW media. Kang (2014) compared different types of media including MS, Woody Plant Medium (WPM) and DKW media for their use in

guayule regeneration and propagation. It was observed that explants cultivated on MS medium resulted in large size calli having multiple shoots but the calli formed on DKW medium were of higher quality, and developed true shoots. The WPM was found to be the best medium for adventitious root formation. Richard *et al*. (2009) evaluated four different types of media (Quoirin and Lepoivre (QL) nutrient, MS, WPM and DKW media) for auxiliary shoot proliferation in pear. They reported DKW media superior to QL, MS and WPM for auxiliary shoot proliferation. The difference in responses of different species may be due to the fact that different genotypes apparently respond differently to different media. The above mentioned formulations for callus induction and regeneration bear great potential and provide baseline information for tissue culture based Brassica improvement programs including future *B. napus* transformation programs.

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