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CALLOGENESIS AND ORGANOGENESIS IN THREE GENOTYPES OF *BRASSICA JUNCEA* AFFECTED BY EXPLANT SOURCE

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Abstract

In vitro studies of *Brassica juncea* is indispensable for evaluating its response to different biotic and abiotic stresses and its improvement through biotechnological techniques. In the present study, we sought to evaluate the *In vitro* response of the three different explants (cotyledons, hypocotyls and roots) of three genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE). The explants were cultured on MS medium supplemented with different combinations of the auxins (NAA and IAA) and cytokinin (BAP and KIN). All the explants responded significantly to all the hormonal concentrations for different parameters. Cotyledons of all the three genotypes were efficient in producing shoots whereas less number of calli were produced by the cotyledons. BAP 2.0 mgL^{-1}/NAA 0.2 mgL^{-1}, BAP 3.0 mgL^{-1}/NAA 0.5 mgL^{-1} and Kin 2.0 mgL^{-1}/IAA 0.2 mgL^{-1} and Kin 3.0 mgL^{-1}/IAA 0.5 mgL^{-1} produced shoots efficiently from the cotyledon explants of the two genotypes, UCD-635 and RL-18. Hypocotyls were found efficient for callus production while their response for shoots formation was slower as more number of days were taken by hypocotyls to produce shoots. Roots were efficient for callus and root formation but neither of the root explants produced shoots on any of the used hormonal concentration. Based on our study, hypocotyls and roots could be the preferred explants when more and vigorous callus formation is required, while cotyledons could be the preferred explants where regeneration is needed such as in genetic transformation experiments.

Introduction

Brassica oilseed species now hold the third position among the oilseed crops and are an important source of vegetable oil (Tohidi-Moghadam *et al*., 2009). Pakistan is spending a huge amount of foreign exchange on the import of edible oil (Mumtaz *et al*., 2001). Indian mustard (*Brassica juncea*) is one of the most important species of the genus *Brassica* (Zhang *et al*., 2006) and is the most common source of edible oil in many Afro-Asian countries (Khatri *et al*., 2005).

In order to increase its yield and seed quality, it is necessary to improve agronomically its important traits, such as herbicide resistance, disease resistance, tolerance to several biotic and abiotic stress factors and fatty acid compositions (Kong *et al*., 2009). It is well known that improvement of plants through conventional breeding methods is slow, time-consuming and labour-intensive. Modern genetic improvement techniques based on molecular genetics and tissue culture has been replacing the conventional breeding methods (Moravcikova *et al*., 2009; Katavic *et al*., 2001).

Regeneration has been optimized through organogenesis and somatic embryogenesis by using different explants (Klima *et al*., 2009) including cotyledons (Ono *et al*., 1994; Bano *et al*., 2010), hypocotyls (Phogat *et al*., 2000; Bano *et al*., 2010), peduncle (Eapen

& George 1997), leaves (Radke *et al*., 1988), flowering internodes (Klimaszewska & Keller, 1985), stem sections (O'Neill *et al*., 1996), immature cotyledons (Turgut *et al*., 1998), microspores (Zhao *et al*., 1996) and protoplasts (Zhao *et al*., 1995). The present study was undertaken to study callus and shoots producing response of different explants of 3 genotypes of *Brassica juncea* viz., UCD-635, RL-18 and NIFA-RAYE cultured on hormones supplemented MS medium.

Materials and Methods

Plant material: Three different genotypes of *Brassica juncea* viz., UCD-635, RL-18 and NIFA-RAYE used in this study were provided by the Institute of Biotechnology and Genetic Engineering (IBGE), NWFP Agricultural University, Peshawar.

Medium for seed germination: Half strength MS (Murashige & Skoog, 1962) medium consisting of 2% sucrose, solidified with 0.8% agar was used for seed germination and seedling growth.

Seed sterilization: For surface sterilization, *Brassica* seeds were washed with tap water and dipped in 70% ethanol for 30 seconds. Then the seeds were treated with sodium hypochlorite solution (1% active chlorine) containing a few drops of TWEEN-20 for 20-25 min followed by rinsing 3-4 times with sterilized water. After sterilization, seeds were cultured for germination on half strength MS medium.

Media for explants culture: MS medium comprising inorganic salts and vitamins supplemented with 3%, sucrose, different concentrations of auxins (indole-3-acetic acid, α-naphthalene acetic acid) and cytokinins (Benzyl amino purine and Kinetin) and solidified with 0.8% agar was used to induce calli and shoots production. All media were sterilized by autoclaving at 121^{o}C and 15 PPS^{-1} for 15 minutes.

Explants preparation: Explants from 3-5 days old seedlings were taken. The basal and apical portions of hypocotyls and roots were discarded and the middle part was cut into 5 mm long segments. Two explants from each cotyledon of a seedling were prepared by cutting in the middle.

Data was recorded for the following parameters:

- Number of explants producing callus on MS medium supplemented with different concentrations of plant growth regulators (PGRs).
- Days to callus formation.
- Days to roots formation.
- Number of explants producing roots.
- Number of explants producing shoots on MS medium supplemented with different concentrations of PGRs.
- Numbers of days to shoot production for the explants cultured on the medium containing PGRs.
- Regeneration efficiency of shoots from explants (calculated by the following formula):

$$RE = \frac{\text{Shoots produced}}{\text{Number of explants}} \text{ X } 100$$

After culturing explants on MS medium supplemented with different concentrations of PGRs, in glass Petri dishes, cultures were incubated at 25°C under 16 h light/8 h dark conditions and observed regularly for callus initiation and/or shoot formation. Pictures of the explants forming callus and shoots, regenerated shoots and rooted shoots were taken. Rooted shoots were transferred to the soil in pots and acclimatized to the greenhouse conditions.

Results

Number of explants producing callus: Mean values for 3 different explants (cotyledon, hypocotyls, and roots) of 3 genotypes (UCD-635, RL-18 and N-R) of *Brassica juncea* cultured on 3 different combinations of each BAP/NAA (BAP 1.0 mgL^{-1}/NAA 0.1 mgL^{-1}, BAP 2.0 mgL^{-1}/NAA 0.2 mgL^{-1}, BAP 3.0 mgL^{-1}/NAA 0.5 mgL^{-}) and Kinetin/IAA (KIN 1.0 mgL^{-1}/IAA 0.1 mgL^{-1},KIN 2.0 mgL^{-1}/IAA 0.2 mgL^{-1}, KIN 3.0 mgL^{-1}/IAA 0.5 mgL^{-1}) showed that maximum values (17.70 and 57.48%) were recorded for the hypocotyls used as an explants source while minimum values were recorded for roots (13.00%) with BAP/NAA and cotyledons (39.85%) with Kinetin/IAA. A significant difference (6.37%) was recorded for number of explants producing callus only with Kinetin/IAA at $p \leq 0.05$ (Table 1). Mean square values for factor (Explants) showed non-significant variation with BAP/NAA but produced highly significant (2102.96) variation with combinations of Kinetin/IAA at $p \geq 0.05$ (Table 2).

Days to callus formation: Mean values for days to callus formation showed that the minimum number of days were for cotyledons (11.85) with Kinetin/IAA and hypocotyls (13.04) with BAP/NAA as explants while the maximum number of days (17.07) was observed for callus formation from root explants on MS medium containing BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1}. The three explants varied significantly from each other where the least significance differences among them were 0.50 with BAP/NAA and 1.95 with Kinetin/IAA at $p \leq 0.05$ (Table 1). Mean square values for days to callus formation were significantly affected by the explants and genotypes (134.33 and 268.59). The effect of interaction of hormones and explants was found highly significant (7.01) with BAP/NAA while non significant results were observed with kinetin/IAA. Interactions of hormones x genotypes, genotypes x explants, and hormones x genotypes x explants significantly affected (24.20, 2.81 and 2.07) the days to callus formation with BAP/NAA and non-significantly affected (182.31, 24.63, 28.59 and 12.24) with all the combinations of kinetin/IAA at $p \leq 0.05$ (Table 2).

Number of explants forming roots: Number of explants forming roots by three explants (cotyledons, hypocotyls, and roots) of the three *Brassica juncea* genotypes showed non-significant variation with BAP/NAA while LSD value for number of explants forming roots was calculated 7.42 at $p \leq 0.05$ with Kinetin/IAA. Maximum value (56.33) was observed for roots as explants while minimum values (46.15) were recorded for cotyledons (Table 1). Mean square values for Number of explants forming roots showed that the effect of explants (618.95) and its interactions (157.16, 483.56, 213.46 and 123.26) were found non-significant with BAP/NAA while with Kinetin/IAA, explants producing roots were significantly (714.86) affected by explants at $p \geq 0.05$ (Table 2).

Table 1. Mean values for different parameters affected by three explants (cotyledons, hypocotyls and roots) of *B. juncea* genotypes.

Explant	XPC	DCF	EFR	DRF	DSI	NPS	RE
	KIN 1.0 mgL^{-1}/IAA 0.1 mgL^{-1},KIN 2.0 mgL^{-1}/IAA 0.2 mgL^{-1}, KIN 3.0 mgL^{-1}/IAA 0.5 mgL^{-1}						
E_1	14.66	16.56b	45.83	14.67b	29.93	21.57a	10.37a
E_2	17.70	13.04a	55.18	17.70a	24.26	18.98 b	3.65 b
E_3	13.00	17.07c	48.72	13.00c	0.00	0.00c	0.00c
LSD		0.50		0.94	3.37	6.94	1.86
	BAP 1.0 mgL^{-1}/NAA 0.1 mgL^{-1}, BAP 2.0 mgL^{-1}/NAA 0.2 mgL^{-1}, BAP 3.0 mgL^{-1}/NAA 0.5 mgL^{-}						
E_1	39.85c	11.85 b	46.15a	16.93b	29.30b	28.7a	1.37a
E_2	57.48a	15.33a	52.56ab	20.67a	23.81a	10.85b	0.88a
E_3	47.92b	13.04c	56.33a	16.81b	0.44 c	0.00c	0.30a
LSD	6.37	1.95	7.42	2.24	2.81	4.95	1.60

Means within a column having the same letters are not significantly different (p = 0.05).
XPC = No. of explants producing callus, DCF = days to callus formation
EFR = number of explants forming roots, DRF = days to roots formation
DSI = days to shoots initiation, NPS = No. of explants forming shoots
RE = regeneration efficiency, AB = interaction of hormones and genotypes
AC = interaction of hormones and explants, ABC = interaction of hormones, genotypes and explants

Table 2. Means square values of different parameters for three *Brassica juncea* genotypes (UCD-635, RL-18 and NIFA-RAYE), using their different explants (cotyledons, hypocotyls and roots), cultured on different levels of BAP/NAA and Kinetin/IAA.

Source	XPC	DCF	EFR	DRF	DSI	NPS	RE
	BAP 1.0 mgL^{-1}/NAA 0.1 mgL^{-1}, BAP 2.0 mgL^{-1}/NAA 0.2 mgL^{-1}, BAP 3.0 mgL^{-1}/NAA 0.5 mgL^{-1}						
Factor A	243.87^{ns}	473.37**	1035.16^{ns}	10.75^{ns}	2788.93**	1740.50**	73.75*
Factor B	226.88^{ns}	134.33**	326.62^{ns}	13.23^{ns}	6.90^{ns}	37.73^{ns}	4.91^{ns}
AB	493.73^{ns}	24.20*	157.16^{ns}	12.53^{ns}	1.19^{ns}	60.53^{ns}	3.64^{ns}
Error	303.06	6.61	313.49	13.51	34.86	264.50	30.36
Factor C	292.02^{ns}	242.25**	618.95^{ns}	153.56**	4519.49**	2629.39**	46.88**
AC	413.87^{ns}	7.01**	483.56^{ns}	3.80^{ns}	1042.67**	1980.32**	50.10**
BC	302.08^{ns}	2.81*	213.46^{ns}	4.90^{ns}	1.75^{ns}	291.08^{ns}	4.38^{ns}
ABC	52.552^{ns}	2.07*	123.26^{ns}	6.17^{ns}	0.43^{ns}	156.25^{ns}	8.05^{ns}
Error	253.16	0.84	427.54	2.94	37.38	158.25	11.39
CV (%)	15.63%	4.61%	14.43%	11.35%	10.51%	3.67%	12.19%
	KIN 1.0 mgL^{-1}/IAA 0.1 mgL^{-1},KIN 2.0 mgL^{-1}/IAA 0.2 mgL^{-1}, KIN 3.0 mgL^{-1}/IAA 0.5 mgL^{-1}						
Factor A	144.75^{ns}	796.70**	116.67^{ns}	198.01^{ns}	2414.03**	1969.78**	190.03**
Factor B	145.74^{ns}	320.11*	3.67^{ns}	235.42^{ns}	12.11^{ns}	35.52^{ns}	0.54^{ns}
AB	279.75^{ns}	182.31^{ns}	324.41^{ns}	227.77^{ns}	24.22^{ns}	5.75^{ns}	4.22^{ns}
Error	474.97	87.63	403.15	143.90	2.81	172.54	23.34
Factor C	2102.96**	268.59**	714.86*	129.79**	4148.92**	5671.59**	92.58**
AC	62.67^{ns}	24.63^{ns}	165.90^{ns}	21.36^{ns}	735.79**	642.24**	55.90**
BC	119.81	28.59^{ns}	206.03^{ns}	25.38^{ns}	23.37^{ns}	251.03*	15.34^{ns}
ABC	121.89^{ns}	12.24^{ns}	106.19^{ns}	16.79^{ns}	30.26^{ns}	15.36^{ns}	2.09^{ns}
Error	133.21	24.63	180.9	16.56	26	1969.78	8.44
CV (%)	12.84%	6.04%	10.03%	8.44%	13.12%	12.85%	11.99%

ns = Non-significant, * = Significant, ** = Highly significant at p≤0.05
Factor A = Hormones, Factor B = Genotype, Factor C = Explants, XPC = No. of explants producing callus, DCF = Days to callus formation, EFR = No. of explants forming roots, DRF = Days to roots formation
DSI = Days to shoots initiation, NPS = No. of explants forming shoots, RE = Regeneration efficiency, AB = Interaction of hormones and genotypes, AC = Interaction of hormones and explants,
ABC = Interaction of hormones, genotypes and explants.

Days to roots formation: Mean values regarding days to roots formation by three explants (cotyledons, hypocotyls and roots) of the 3 *Brassica juncea* genotypes was found significantly different with all the hormonal combinations used with least significant differences of 0.94 on MS medium containing BAP/NAA and 2.24 on MS medium containing Kinetin/IAA. Minimum values (13.00) were recorded for roots used as an explant on MS medium containing BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1} while the maximum number of days (20.67) to produce roots was observed for hypocotyls used as explants with kinetin/IAA at $p\leq0.05$ (Table 1). Mean square values for days to roots formation showed highly significant effects (153.56 and 129.79) for factor (explants) and non-significant effects for its interactions at $p\geq0.05$ (Table 2).

Days to shoots initiation: The ability of shoots formation of all the three explants (cotyledons, hypocotyls and roots) had significant differences among them on all the combinations used with LSD values of 3.37 and 2.81 respectively. Maximum mean value (29.93 and 28.30) was observed for cotyledons used as explants of the three *Brassica juncea* genotypes, while roots used as explants of three genotypes failed to produce shoots on all the hormonal concentrations of BAP/NAA and Kinetin/IAA. Hypocotyls were found efficient among the three explants used with mean values of 23.81 and 24.26 at $p\leq0.05$ (Table 1). Mean square values for days to shoots initiation were highly significantly effected for the factor explants (4519.49 and 4148.92) and its interactions with hormones (1042.67 and 735.79) at $p\leq0.05$ (Table 2).

Number of explants forming shoots: Maximum number of shoots (28.70 and 21.57) was observed from cotyledons of the three genotypes on all the hormonal concentrations used while roots used as explants was observed non-responsive to shoot formation. The least significant differences recorded were 6.94, 1.60 and 4.95 at $p\leq0.05$ (Table 1). Highly significant mean square values for number of explants produced shoots were observed for explants (746.88 and 5671.59). Similarly the interaction of hormones x explants was also recorded highly-significant (1980.32 and 642.24) at $p\geq0.05$ (Table 2).

Regeneration efficiency: Maximum regeneration efficiency (10.37) was recorded for cotyledons used as an explant on MS medium containing BAP/NAA while roots has no response for regeneration. The least significant differences recorded for regeneration efficiency were 1.86 and 1.60 at $p\leq0.05$, showing that the regeneration efficiency of these 3 explants varied significantly from each other (Table 1). Mean square values for regeneration efficiency by the explant (746.88 and 692.58) and its interaction with hormones (50.10 and 55.90), also showed highly significant variation at $p\geq0.05$ (Table 2).

Discussion

In this study the effect of 3 different explants (cotyledon, hypocotyls and roots) on callogenesis and organogenesis was studied in detail in 3 genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE). The explants were cultured on MS medium supplemented with different concentration of hormones and incubated at 25^{o}C under 16 h light/8 h dark conditions and observed regularly for callus initiation and shoot formation. Callus induction and growth started from cut ends of the explants cultured on MS medium containing different concentration of auxins and cytokinin and eventually extended all over the explants, especially, in case of hypocotyls and roots.

Approximately, three weeks after culturing, the explants of hypocotyls and roots almost completely converted into callus. In case of hypocotyls one end of the explant developed more callus than the other end. On cotyledon, callus formed on the cut surfaces around and rarely on the middle of the explant. In general, a high percentage of explants produced calli. Similar results have been described in other reports (Muhammad *et al*., 2002; Moghaieb *et al*., 2006). However, no significant differences in callus induction between the cultivars were observed (Moghaieb *et al*., 2006) (Fig).

Time taken by the explants of the 3 genotypes to produce callus was found different. Such results can be attributed to the genetic differences of the genotypes. Genotypic influence on *In vitro* morphogenesis in *Brassica* has been documented previously (Kamal *et al*., 2007; Khehra & Mathias, 1992). Such reports indicate that the developmental processes reflected by *In vitro* response are genetically controlled.

Callus from the cotyledon was found more regenerative than that from the other two explants. The explants also produced white, hairy and dense adventitious roots. Adventitious root production from the cotyledon explants was found more profusely (Fig A, B & D).

Explant types (cotyledons, hypocotyls and roots) were different in their ability to produce shoots. Significant differences in shoot regeneration of these explants were observed when the results were analyzed using one way ANOVA at $\alpha<0.05$. In contrast, Zhang & Bhalla (1999) obtained non-significant differences in the response the three explants (cotyledons, hypocotyls and roots) of *Brassica napus.* Such different results might be attributed to the different species and hence, the different genetic potential of regeneration from the three explants. During this study a maximum number of shoots (> 40) were produced from UCD-635. However, mean separation by MSTATC showed that the 3 genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE) were not statistically different in their shoot regeneration capacity.

Cotyledons were first to initiate callus and shoot production while root explants were slow in culture, indicating that cotyledons might be more suitable for transformation experiments. This difference in the response of the genotypes may be due to different requirements of the explants for optimal callus and shoot regeneration. Khehra & Mathias (1992) studied the importance of explant type, genotype and growth hormone regime on shoot regeneration of a number of *B. napus* genotypes. Their study found that the most important factors for shoot regeneration were explant type and genotype and the influence of the hormone regime was negligible.

Hypocotyls were efficient in callus formation followed by the roots and cotyledons. Hypocotyls needed longer callus phase for shoot production as compared to the cotyledon. In addition, hypocotyls produced shoots on MS medium supplemented with higher concentration of PGRs (BAP 3 mgL^{-1} + NAA 0.5 mgL^{-1}). The contribution of endogenous level of hormones in callus formation and shoot production cannot be neglected. Different explants need varied doses of exogenous hormones for callus and shoots production.

Based on our study, hypocotyls and roots could be the preferred explants when more and vigorous callus formation is required, especially in studying the effect of different biotic and abiotic stresses under *In vitro* conditions. However, cotyledons explants could be the preferred explants for genetic transformation experiments because of its highest regeneration efficiency.

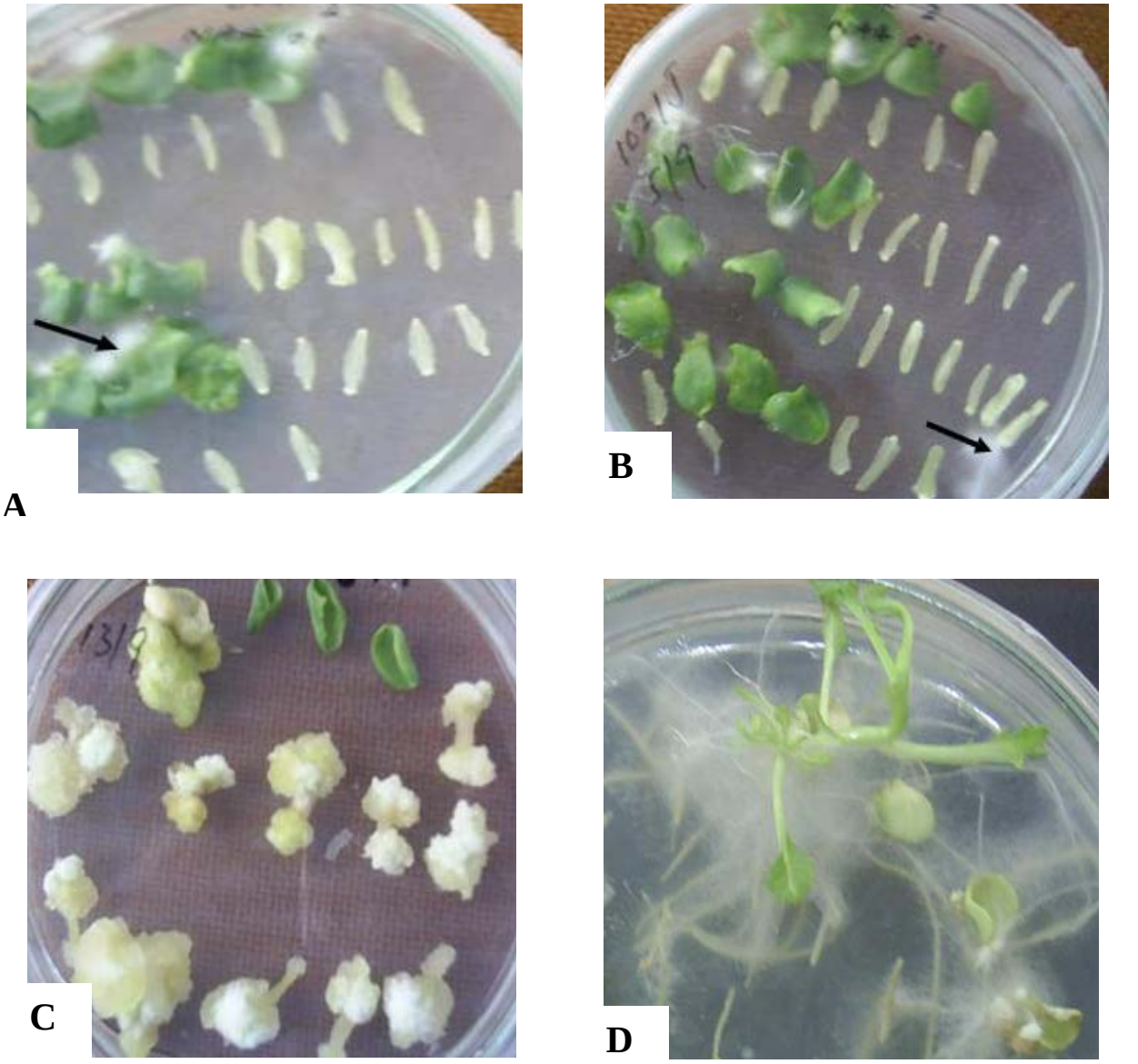

Fig. 1. Callus, Shoots and adventitious roots production by cotyledon and hypocotyls of *Brassica juncea.*
A & B. Callus and root production by cotyledon (arrow head) and hypocotyls (arrow head) 18 days after culture.
C. Enlarged callus formed by hypocotyls of genotype RL-18 on MS medium supplemented with kin 2.0 mgL^{-1}/IAA 0.2 mgL^{-1}.
D. Shoots produced by cotyledon of *Brassica juncea genotype,* UCD-635 on MS medium supplemented with BAP 2.0 mgL^{-1} + NAA 0.2 mgL^{-1}.

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