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Plant regeneration and *Agrobacterium*-mediated transformation in Indian mustard (*Brassica juncea*)

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Abstract

Using seedling explants, a highly efficient and reproducible plant regeneration and transformation system was developed in *Brassica juncea* genotypes RH-406 and RH-555. Hypocotyl and cotyledon explants excised from *in vitro*-grown 5-days-old seedlings were cultured on MS medium supplemented with different concentrations and combinations of growth regulators. Cotyledon explants grown on MS medium supplemented with 1 and 2.5 mg/L BAP produced higher per cent shoot formation in genotypes RH-555 (60.3±4.19) and RH-406 (64.9± 1.42), respectively. Among the eight rooting media used for root formation in regenerated shoots, maximum rooting response was obtained on MS medium supplemented with 0.2 mg/L NAA. Among the regenerated plants, 81.8 % in RH-406 and 67% in RH-555 survived when transferred on sand and soil in 1:1 mixture in pots. Transformation protocol was developed in genotype RH-406 using GUS reporter gene and hypocotyls and cotyledon explants. Histochemical GUS assay showed that cotyledon and hypocotyl explants respectively, had 75% and 80% transient GUS expressions.

Key words: *Brassica juncea*, GUS, regeneration and transformation

Introduction

Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is one of the most important species of the genus *Brassica* (Zhang *et al*., 2006) as a source of edible oil in many Afro-Asian countries (Khatri *et al*., 2005). India holds a leading position in world rapeseed-mustard economy with 2nd and 3rd rank in area and production, respectively. It is an amphidiploid, originated from hybridization between *B. rapa* and *B. nigra*. Regeneration is an important component of plant genetic engineering for crop improvement. Plant regeneration in *Brassica* species is highly genotype dependent (Ono *et al*., 1994; Zhang *et al*., 1998; Mollika *et al*., 2011; Takahashi *et al*., 2012). For successful development of transgenics, plant regeneration and transformation procedures must be very efficient and reproducible. This is needed to produce a large number of transformants for selection of most suitable transgenic plants for varietal development. Indian mustard production is adversely affected by several pests and pathogens for which genes are not available in wild species to improve these traits. Several valuable genes are available in cultivated *Brassica* species which have potential to improve these traits. The present investigation was, therefore, carried out to develop an efficient and reproducible regeneration system and optimization of transformation in elite Indian mustard cultivars.

Materials and Methods

Plant Regeneration

Seeds of *B. juncea* genotypes RH-406 and RH-555 were obtained from Oilseeds Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. For raising seedlings, seeds were washed with tween 20, surface sterilized with 0.1% mercuric chloride for 5-7 minutes, rinsed thrice with sterilized distilled

water, aseptically placed on Murashige and Skoog (1962) (MS) basal medium, and incubated at 25±1°C under 16 hr light and 8 hr dark photoperiod. Cotyledon and hypocotyl explants from 5-days-old seedlings were cut and placed on petri plates containing MS media with different concentrations and combinations of growth regulators. The shoots thus produced were transferred on MS media containing 0.2 mg/L NAA for rooting. The plantlets were transferred to sterilized soil and sand mixture (1:1) in the pots.

Plant Transformation

Agrobacterium tumefaciens strain EHA 105, harbouring pCAMBIA 2301 vector was used to optimize factors influencing the transformation experiments. This binary vector contains GUS as reporter gene. The bacterial strain from the glycerol stock culture streaked on (Luria Bertani) LB-agar plates containing kanamycin (50mg/L) and rifampicin (10mg/L) supplemented media were incubated at 28°C for raising fresh bacterial culture. A single bacterial colony was inoculated into 10 ml LB broth with 50 mg/L kanamycin, incubated at 28°C overnight on a rotary shaker at 140 rpm, diluted 20 fold, and incubated again for 3-4 hr at 28°C. The bacterial cultures of different optical densities (0.5, 1, 2) were used for co-cultivation with explants for different time intervals, and histochemical GUS assays. *Agrobacterium*-infected and non-infected control explants were stained with X-gluc staining solution, and incubated at 37°C for 24h, 48h and 72 h for GUS assays.

Results and Discussion

Using hypocotyl and cotyledon explants from 5-days-old seedlings, an *in vitro* plant regeneration system for *B. juncea* genotypes RH-406 and RH-555 was developed on MS medium supplemented with varying concentrations of 6-Benzylaminopurine (BAP), Kinetin and Thidiazuron (TDZ) cytokinins alone or in combinations, and ±- Napthalene Acetic Acid (NAA) auxin. Calli and single or multiple shoots developed after about two weeks were transferred on to rooting media, and the plantlets so developed were further transferred into pots in the green house.

Cotyledon explants

Highest (64.9±1.4) and lowest (23.8±1.2) per cent shoot regenerations in genotype RH-406 (Fig. a) were observed in MS medium supplemented with 2.5 mg/L BAP and 5.0 mg/L Kinetin + 0.2 mg/L NAA, respectively (Table 1). Similarly, in genotype RH 555, MS medium supplemented with 1.0 mg/L

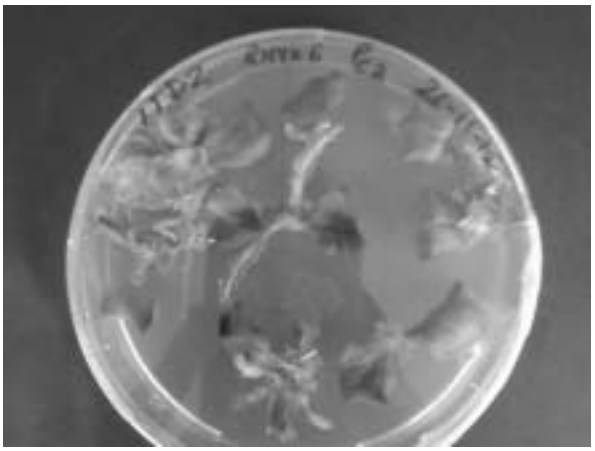

Fig. a Shoot formation in cotyledon explants of *B. juncea* genotype RH-406 on MS medium supplemented with 1 mg/L TDZ

BAP and 5.0 mg/L Kinetin + 0.2 mg/L NAA induced highest (60.3±4.2) and lowest (12.6±1.3) shoot regeneration, respectively (Table 1). Gaur *et al.* (1996) also observed highest shoot regeneration on MS medium supplemented with 2.0 mg/L BAP and 0.2 mg/L IAA in Indian mustard genotypes RH-30 and RH-8812. Bano *et al.* (2010) also reported maximum shooting (22.3) in *Brassica juncea* genotypes UCD-635, RL-18 and NIFA RAYE on MS medium supplemented with 3.0 mg/L BAP and 0.3 mg/L NAA and 3.0 mg/L Kinetin and 0.3mg/L NAA. Highest number of shoot regeneration was also reported in Broccoli on medium supplemented with a combination of 1 mg/L BAP and 1.5mg/L IBA (Farzinebrahimi *et al*., 2012).

Hypocotyl explants

Eighteen media combinations were evaluated for shoot regeneration in hypocotyl explants of *B. juncea* genotypes RH-406 and RH-555 (Table 2). Hypocotyl explants produced shoots only in 6 out of 18 MS media combinations (Table 2). Highest per cent shoot formation (58.1±1.5) in genotype

Table 1: Effect of MS-modified media on shoot regeneration in cultured cotyledon explants of *Brassica juncea* genotypes RH-406 and RH-555

MS-modified media	Per cent Shoot regeneration	
	RH 406	RH 555
MS + BAP (1.0 mg/L)	46.3±2.7	60.3±4.2
MS + BAP (2.5 mg/L)	64.9±1.4	49.6±5.3
MS + BAP (5.0 mg/L)	33.2±1.85	22.6±2.55
MS + BAP (1.0 mg/L) + NAA (0.2 mg/L)	42.3±2.85	40.4±1.75
MS + BAP (2.5 mg/L) + NAA (0.2 mg/L)	42.4±1.74	34.6±2.50
MS + BAP (5.0 mg/L) + NAA (0.2 mg/L)	27.2±2.07	21.4±2.21
*MS + Kin (1.0 mg/L)	31.0±4.37	15.1±1.98
MS + Kin (2.5 mg/L)	29.2±2.07	16.4±1.09
MS + Kin (5.0 mg/L)	28.6±4.24	23.0±1.08
MS + Kin (1.0 mg/L) + NAA (0.2 mg/L)	23.9±1.18	13.9±2.16
MS + Kin (2.5 mg/L) + NAA (0.2 mg/L)	29.2±2.07	12.8±1.40
MS + Kin (5.0 mg/L) + NAA (0.2 mg/L)	23.8±1.18	12.6±1.28
MS + TDZ (1.0 mg/L)	35.0±1.11	25.0±1.95
MS + TDZ (2.5 mg/L)	35.0±1.85	24.7±1.58
MS + TDZ (5.0 mg/L)	36.8±3.12	24.8±3.25
MS + TDZ (1.0 mg/L) + NAA (0.2 mg/L)	37.0±1.40	22.8±3.13
MS + TDZ (2.5 mg/L) + NAA (0.2 mg/L)	35.2±0.83	27.8±5.87
MS + TDZ (5.0 mg/L) + NAA (0.2 mg/L)	35.0±1.06	24.9±1.16

*Kin- Kinetin

Table 2: Effect of MS-modified media on shoot regeneration in cultured hypocotyl explants of *Brassica juncea* genotypes RH-406 and RH-555

MS-modified media	Per cent Shoot regeneration	
	RH-406	RH-555
MS + BAP (1.0 mg/L)	58.1±1.5	4.0±0.1
MS + BAP (2.5 mg/L)	50.3±0.2	6.9±0.2
MS + BAP (5.0 mg/L)	10.4±0.1	0
MS + BAP (1.0 mg/L) + NAA (0.2 mg/L)	20.0±0.5	2.2±0.1
MS + BAP (2.5 mg/L) + NAA (0.2 mg/L)	40.0±0.5	3.4±0.4
MS + BAP (5.0 mg/L) + NAA (0.2 mg/L)	30.2±0.5	0
*MS + Kin (1.0 mg/L)	19.1±1.1	0
MS + Kin (2.5 mg/L)	34.3±0.2	0
MS + Kin (5.0 mg/L)	10.1±1.0	0
MS + Kin (1.0 mg/L) + NAA (0.2 mg/L)	12.1±0.2	0
MS + Kin (2.5 mg/L) + NAA (0.2 mg/L)	28.4±0.2	0
MS + Kin (5.0 mg/L) + NAA (0.2 mg/L)	10.1±0.1	0
MS + TDZ (1.0 mg/L)	38.1±0.2	7.6±0.2
MS + TDZ (2.5 mg/L)	34.8±0.4	0
MS + TDZ (5.0 mg/L)	30.1±0.3	0
MS + TDZ (1.0 mg/L) + NAA (0.2 mg/L)	21.1±0.2	4.2±0.1
MS + TDZ (2.5 mg/L) + NAA (0.2 mg/L)	18.8±1.2	0
MS + TDZ (5.0 mg/L) + NAA (0.2 mg/L)	16.4±1.3	0

*Kin-Kinetin

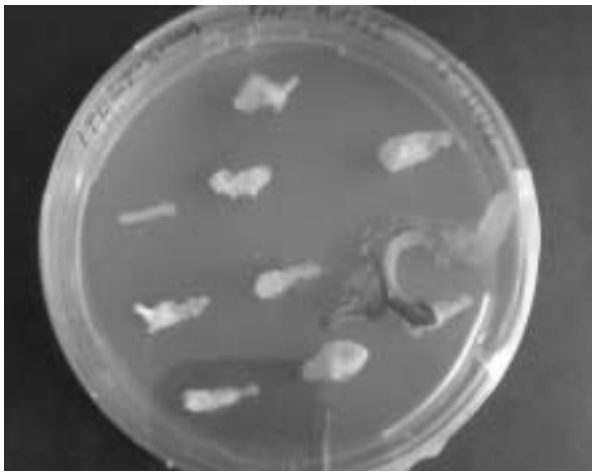

Fig. b Shoot formation in hypocotyl explants of *B. juncea* genotype RH-555 on MS medium fortified with 1 mg/L TDZ + 0.2 mg/L NAA

RH-406 was observed on MS medium containing 1.0 mg/L BAP. In genotype RH-555, highest percent shoot formation (7.6±0.2) occurred on MS medium supplemented with 0.1mg/L TDZ (Fig. b). These results are in close conformity with the studies of Pavlovic *et al.* (2010) in *B. oleracea* varieties (red cabbage, broccoli, Savoy cabbage and cauliflower) who also reported hypocotyl explants with a minimum regeneration potential of 75% and producing 3.5-7.4 shoots per explant on MS medium supplemented with 1.0mg/L BAP. In *B. napus*, highest per cent shoot formation was also observed on MS medium supplemented with 2.0 mg/L BAP and 0.2 mg/L NAA (Ali *et al.*, 2007).

Rooting

Eight rooting media , including MS basal, half strength MS basal and MS basal with two rates of each of IBA, NAA and IAA auxins, were evaluated for inducing roots in regenerated shoots (Table 3). Basal MS medium, although, induced rootings in regenerated shoots to the extent of 60.3 and 40.3 per cent in genotypes RH-406 and RH-555, respectively, addition of both rates of auxin NAA, significantly increased rootings in both genotypes (Table 3). MS medium supplemented with 0.2mg/L NAA appears to be the optimum rate, because this combination induced the highest rootings in the range of 93.4% and 90.7% respectively, in genotypes RH-406 and RH-555; addition of 0.5mg/L NAA, also significantly increased rootings over the basal MS medium (Table 3). All other rooting media containing two

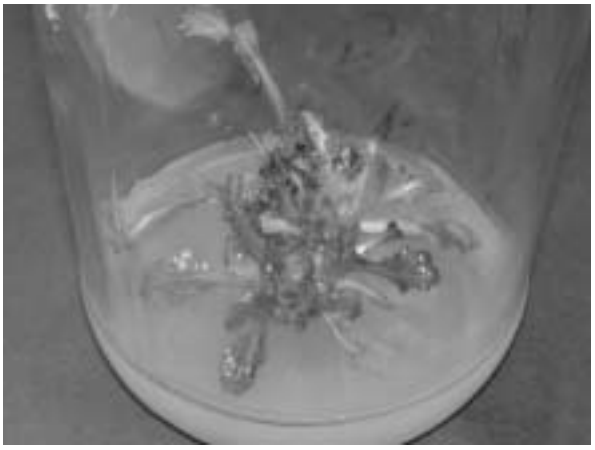

Fig. c: Multiple shoot formation

Table 3: Per cent root formation in regenerated shoots of *Brassica juncea* on MS-modified media with three auxins

Rooting media	Per cent root formation	
	RH-406	RH-555
MS Basal	60.3±1.2	40.3±1.2
Half strength MS Basal	18.5±1.1	15.3±1.6
MS + IBA (0.2mg/L)	10.5±1.1	8.1±1.9
MS + IBA (0.5 mg/L)	16.3±1.5	12.1±1.0
MS + NAA (0.2mg/L)	93.4±0.8	90.7±1.5
MS + NAA (0.5 mg/L)	77.7±0.3	67.7±1.2
MS + IAA (0.2 mg/L)	20.9±1.6	17.3±1.8
MS + IAA (0.5 mg/L)	19.3±1.2	14.8±0.6

rates of each of IBA and IAA did not induce much rootings as their values were similar to those observed in half strength MS basal medium. Results of our present studies are in agreement with the studies of Naibo *et al.* (2009), and Khan *et al.* (2009), but not with the results of Ravanfar *et al.* (2009) who found MS medium supplemented with 0.2mg/L IBA to be the most effective rooting medium for *B. oleracea* var. *Broccoli* cv. Green marvel. As in our present studies, Naibo *et al.* (2009) in their plant regeneration system with *B. oleracea* var. Multiceps, and Khan *et al*. (2009) in the *Agrobacterium*-mediated genetic transformation experiments with *Brassica* species Tori-7, reported highest rootings in MS media supplemented with 0.2mg/L and 0.5mg/L NAA, respectively.

Effect of pre-culturing, agrobacterium-infection and co-cultivation for transient GUS expression

In genotype RH-406, maximum transient GUS expressions, in both hypocotyl (80%) and cotyledon (75%) explants, were observed with 2-day-pre-cultured explants inoculated with *Agrobacterium* for 30 minutes and co-cultivated for 72hr (Fig. g, h). Yadav *et al.* (2003) also demonstrated pre-culturing of explants, and co-cultivation duration of 72hr as essential requirements for efficient transformation in *Brassica* species. Similarly, Khan *et al.* (2009) also reported GUS expression of 80% in two varieties of *Brassica* explants precultured for 72hr.

Using hypocotyls and cotyledons explants, an efficient plant regeneration and *Agrobacterium*-mediated transformation protocol was optimized in two elite Indian mustard genotypes RH-406 and RH-555 (Figs. e, f, g and h). Histochemical transient GUS expression assays showed high percentage of gene expression. The protocol developed in the present study can be exploited for introducing valuable genes through *Agrobacterium-mediated* transformation in Indian mustard.

Fig. d: Regenerated plant of *B. juncea* (RH-406) transferred into pot covered with polythene bags

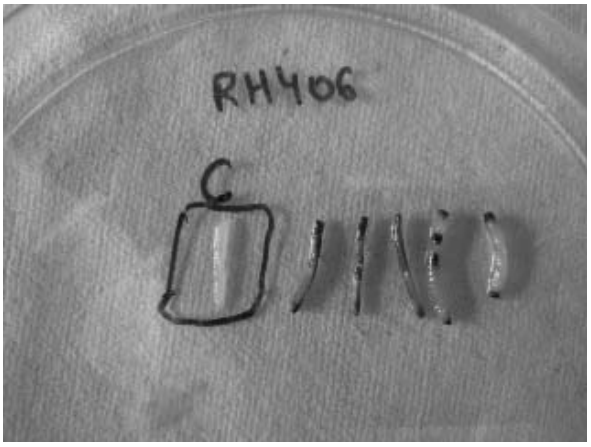

Fig. e: Transient *GUS* expression in hypocotyl explants of *B. juncea* genotype RH-406

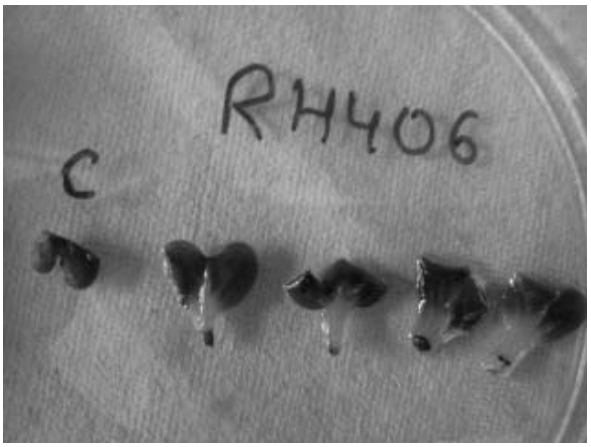

Fig. f: Transient *GUS* expression in cotyledon explants of *B. juncea* genotype RH-406

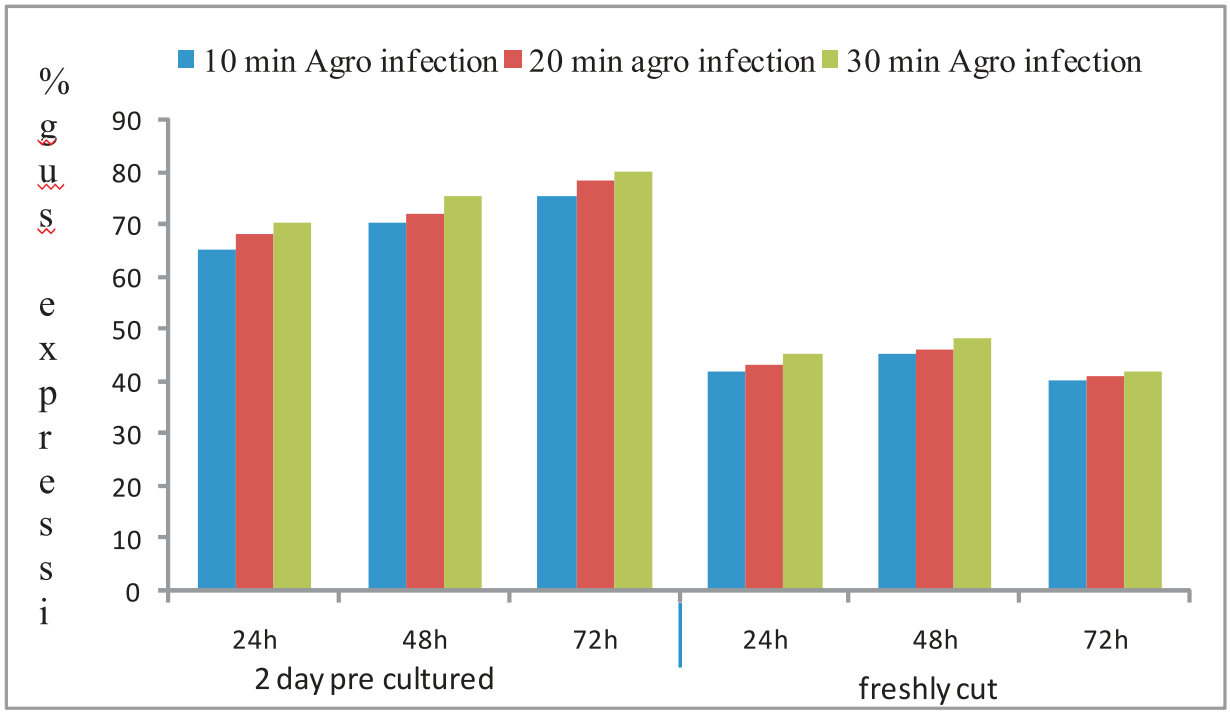

Fig. g: Effect of pre-culturing, Agro infection, and co-cultivation on transient *GUS* expression in hypocotyl explants of *B. juncea* genotype RH-406

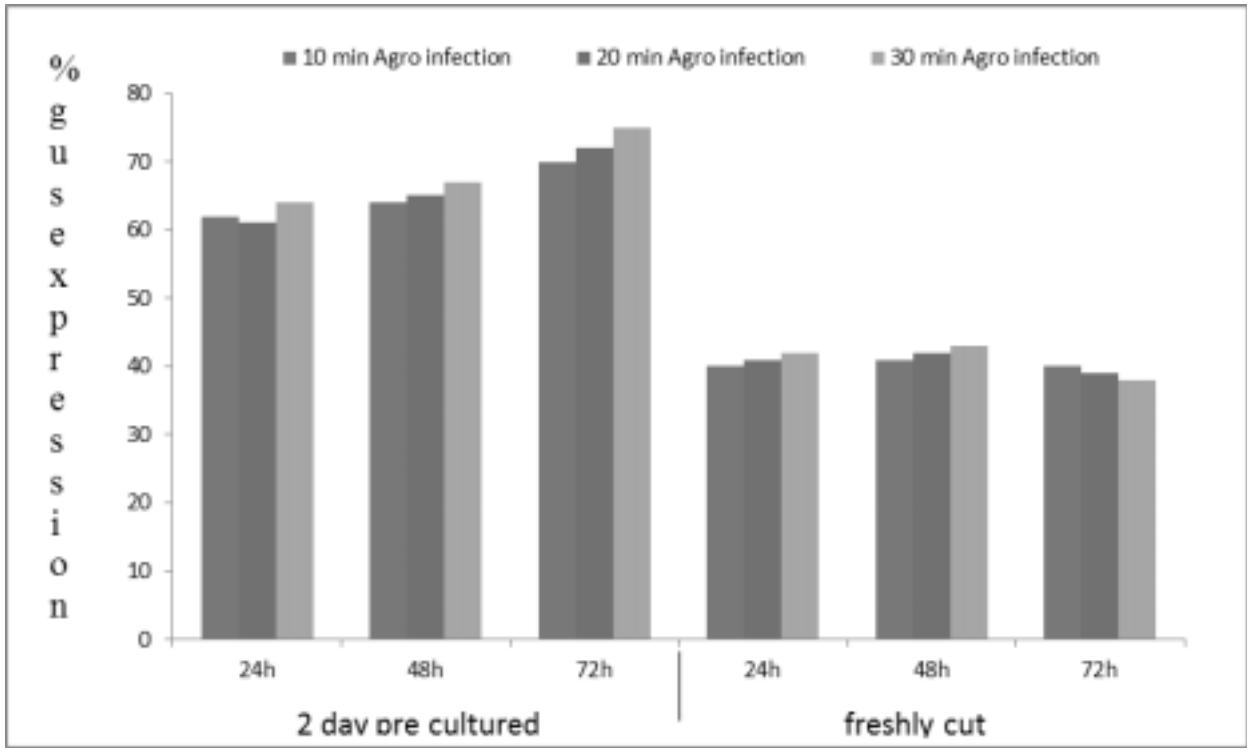

Fig. h: Effect of pre-culturing, Agro infection, and co-cultivation on transient *GUS* expression in cotyledon explants of *B. juncea* genotype RH-406

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