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Callus induction in wide cross of *Brassica*

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ABSTRACT

Utilisation of naturally occurring variability of cultivated species in Brassica is limited due to its narrow genetic base. The pre-breeding approaches for introgression of crucial genes from wild to cultivated species faces barriers in generation of wide hybrids. In such a situation, embryo rescue has proved to be one of the best tools for generation of distant hybrids. The present study was carried out with the objective to standardise the media composition for the rescue of hybrid embryo obtained through the inter-specific crosses between Brassica fruticulosa (FF, 2n =16) and Brassica rapa (AA, 2n = 20). Pistils of crosses obtained after 3 to 5 days of pollination were used as explants. Out of three media compositions MS media supplemented with casein hydrolysate and benzo-aminopurine reflected the best result for ovary and ovule culture to revive the embryo. The well established pistils showed more than eighty per cent swelling of cells. The axially organized growth of cells was observed in the swelled explants and later it was converted into a shapeless bulk of living matter. After 21 days of inoculation, growth of cell mass appeared in the form friable and greenish cream callus. About sixty percent callogenesis was observed. The optimised media compositions may be useful in generation of wide cross of Brassica and related crops.

Key words: *Brassica fruticulosa and Brassica rapa, Callogenesis, Embryo rescue, Mustard*

INTRODUCTION

The oleiferous *Brassica* belongs to *Brassicaceae* family and is used as cooking oil, constituent of vegetable and condiment production in the world. Most of the cultivated varieties are susceptible to biotic and abiotic stresses, which results in gap between demand and supply chain of the commercial products. Wild species of *Brassica* are treasurers of many favourable genes that support their breeding fitness to complete their life cycle even under stress conditions.

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Introgression of these favourable genes from wild *spp*. to cultivated ones faces either pre-fertilization or post fertilization crossabilty barriers mostly due to unparallel ploidy levels or gene pools. Many researchers have used embryo culture, ovary culture and ovule culture (Bajaj, Y.P.S. 1986; Agnihotri, A. 1990; Churungu, B. 1999; Chandra, A. 2004; Ji-Peng Chen, 2011; and Atri, C. 2012) to enhance the wide hybridization in *Brassica*. However, the detailed or clear-cut optimised protocol for rescue of embryo in *Brassicas* is poorly reported and needs to pay attention towards it. In this study a wild crucifer; *Brassica fruticulosa* (Native to the Mediterranean coast) was used for wide hybridization with *Brassica rapa* as a bridge species with the aim to introgress the aphid resistant gene (s) in cultivated varieties of Indian mustard. *B. fruticulosa* has found to be tolerant to cabbage aphid (Ellis and Farell 1995; Pink *et al*. 2003) and is also known to be resistant to mustard aphid (Atri, C. *et al*; 2012). The present experiment was laid with objective to optimize the media composition for the rescue of hybrid embryo obtained through a wide cross of *B.fruticulosa* and *B. rapa.*

MATERIALS AND METHODS

A. Selection of parents for wide crosses: In the present study, a wild species *Brassica fruticulosa* and a cultivated species *Brassica rapa* were used as seed and pollen parent respectively to raise the wide hybrid. Seeds of these species were procured from reputed source and sowed in Rabi for its multiplication, adaptation, genetic purity maintenance and morphological characterization along with the evaluation for aphid tolerance/susceptible response under controlled field conditions of BAU, Sabour, Bhagalpur. In subsequent season, their pure seeds were sowed in crossing block in four different dates with one week of intervals to synchronize the flowering period and prolong availability of pollen and seed parent. Standard agronomical practices were employed to raise a healthy crop.

B. Emasculation of selected plants: After plants were reached at flowering stage, individual plants selected visually on the basis of optimal morphological developments. Flowers of selected pollen parent (*Brassica rapa*) were covered with butter paper bag to maintain the purity of pollen grains. Similarly seed parent (*Brassica fruticulosa)* was also selected visually on the basis of optimal morphological developments. Hand emasculation was employed using forceps in selected flowers of female plants. The immature and over-matured buds were discarded due to non receptive behavior and already selfed event of stigma respectively. All the possible precautions were followed to avoid the stigmatic injury and purity of the desired cross combinations. The emasculated flowers were covered with butter paper bag and marked with tags.

C. Pollinationof selected female plants: In congenial environment pollen loaded fresh open flowers from selected male plants were collected in a Petri plate and covered to avoid foreign pollen. The dehiscing anthers of collected flowers were touched manually with gentle rubbing on stigma of emasculated female flowers. After pollination, plants were again covered with butter paper bag and marked with tags.

D. 2, 4-Dichlorophenoxyacetic acid treatments: After 24 hours. of pollination, the pistil of each crosses were gently treated with approximately equal amount of 0.1% 2, 4-Dichlorophenoxyacetic acid (2, 4-Dichlorophenoxyacetic acid is an organic compound with the chemical formula $C_8H_6Cl_2O_3$ and its solution was prepared in ethanol) with the help of sterilized cotton dipped inside the 2, 4-D solution.

E. Disinfection of pistils: Pistils obtained 2 to 6 days after pollination, were collected in Petri-plate containing distilled water. Pistils

were disinfected through 0.1% mercuric chloride with gentle shaking in autoclaved Petri-plates for 10 minute inside the laminar air flow followed by 5 times washing with autoclaved distilled water to removes the residual mercuric chloride.

F. Growing in culture medium: Ovary culture followed by ovule culture was adopted for the rescue of embryos**.** Disinfected five pistils per culture bottle were put aseptically in Murashige and Skoog (MS, medium (1962) as a basal medium supplemented with casein hydrolysate and banzoaminopurine. Three types of media compositions were used namely M1 [Full MS media+3% sucrose], M2 [Full MS + Caesine hydrolysate {$0.5gl^{-1}$} +3% sucrose] & M3 [Full MS media +Caesine hydrolysate {$0.5\ mgl^{-1}$} + Banzoaminopurine {$0.5\ mgl^{-1}$} +3% sucrose].

Pistils were incubated under dark conditions for 2-3 days to overcome the stress generated during preparation of aseptic pistil. Then pistils were shifted under white light conditions for 12 h in a circadian clock fashion. Similarly,some of the excised pistils whose ovules were extracted manually with needle were also disinfected inside the laminar air flow and were incubated followed by keeping in white light treatments. Swelling was observed after 5-8 days of establishment of culture. Contrary there was no remarkable swelling observed in excised pistils devoid of ovules in the media used.

G. Sub-culturing of pistils: The swelled portion of pistils having swelled ovules were dissected vertically at place between the two consecutive ovules and then dissected ovules were sub culture in MS medium supplemented with benzoaminopurine and again kept under for 12 h in a circadian clock fashion.

H. Generation of callus: After 15 to 20 days of light treatment the whitish and greenish un-differentiated mass of callus in swelled dissected ovules was observed. In later developmental stage, same callus becomes greenish in same full MS medium supplemented casein hydrolysate and benzoaminopurine and kept under 12 h light and 12h of dark in a circadian clock fashion. In due course of time sub culturing of callus in same media was done.

I. Statistical analysis: All the Statistical analysis was done by using the software Graph Pad Prizm 5

RESULTS AND DISCUSSION

A. Pistil length was observed more when treated with 2, 4-Dichlorophenoxyacetic acid after 3 days, 5 days and 7 days of pollination: A total of 2178 buds were attempted for emasculation in different dates. Fertilized pistils of different age (2 days, 4 days, 8 days and 10 days after pollination) were taken for embryo rescue. The maximum number of pistils i.e. 65 was retained after two days of pollination (Fig.1d). The number of retained pistils thereafter decreases sequentially from 4 days to 10 days after pollination from about 55 to 10 in numbers respectively. This result clearly indicates that days after pollination play pivotal role to retain the pistils after pollination. A significant differences of total no. of pistils retained in treated vs. non- treated with 2-4-D was also observed (Fig.1e).

Many pistils retained after *fruticulosa-rapa* crossing significantly reflected the effect of 2, 4-Dichlorophenoxyacetic acid on pod length and thickness, resulted in increased pod length & thickness as compared to untreated pistils after 3 days, 5 days and 7 days of pollination (Fig.1a-a', b-b' & c-c'). With the use of paired t-test the significant difference of pistil size between untreated and treated was analysed. The results showed more thickness of pistil, with increasing size irrespective of individual crosses and in all three types of media compositions (M1, M2 & M3). The highest increase in sizes was observed in M3 after 3 days, 5 days and 7 days of pollination respectively. It can be co-related that the increased in pod length might be because of

some cell inducing common mechanism of polarity for this longer pod length development. Polarization of cells and tissues is crucial for plant morphogenesis, because the emerging morphogenetic gradients provide the basis for differential genome activity at various stages of plant development. The polarity axes are established at the stage of zygote, extending to the developing embryo, and they "vectorize" subsequent plant growth and development.

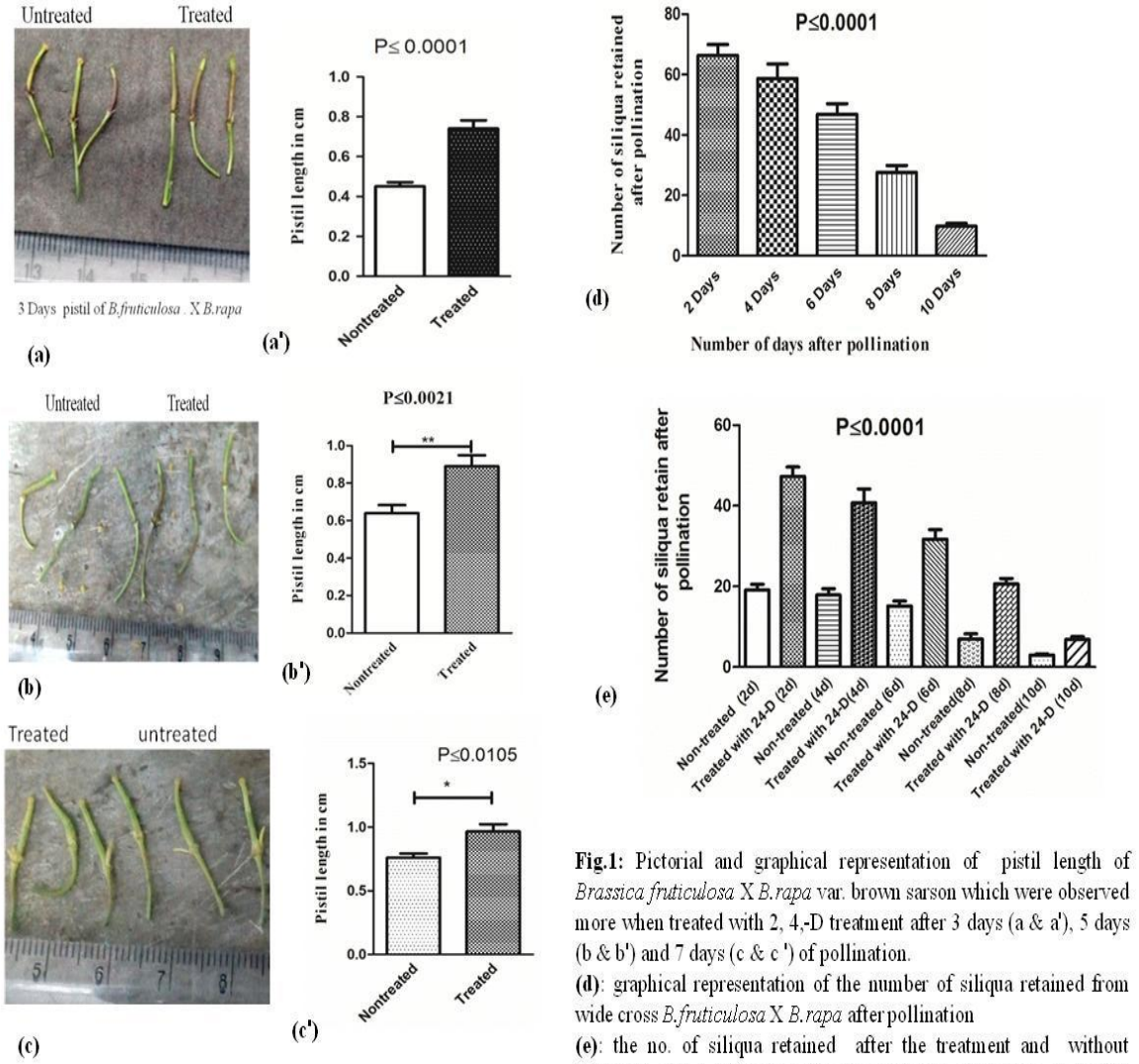

Fig.1: Pictorial and graphical representation of pistil length of *Brassica fruticulosa* X *B.rapa* var. brown sarson which were observed more when treated with 2, 4,-D treatment after 3 days (a & a'), 5 days (b & b') and 7 days (c & c ') of pollination.
(d): graphical representation of the number of siliqua retained from wide cross *B.fruticulosa* X *B.rapa* after pollination
(e): the no. of siliqua retained after the treatment and without treatment of 2, 4,-D treatment in different days of intervals (2,4,6,8 & 10 days) after pollination

B. Establishment of retained pistils in MS media with different additive components: It is well understood that establishment of any explants in respective appropriate media is first essential step towards understanding the tissue culture responses in different media. In the present experiment the media M3 showed significantly highest establishment percentage as compared to M2 and M1 media respectively (Fig.2a-a', b-b' & c-c') under 5-6 days of light treatment. Hence, result clearly suggested the crucial role of media composition in establishment of pistils using different Media and additives.

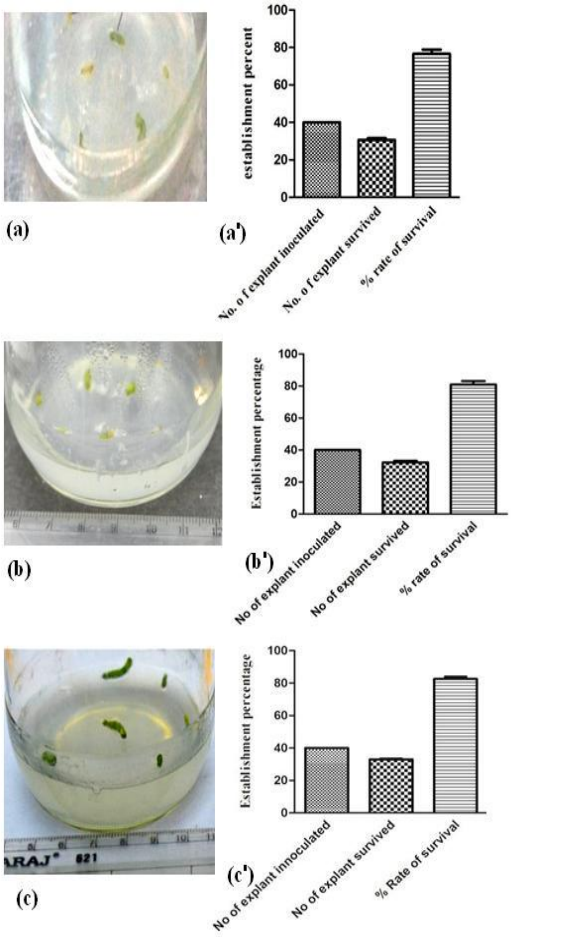

Fig.2: Pictorial and graphical representation of establishment and establishment per cent respectively of *Brassica fruticulosa* X *B.rapa* var. brown sarson observed in medias M1 (a & a'), M2 (b & b') and M3 (c & c ') under 5 to 6 days of light treatment.

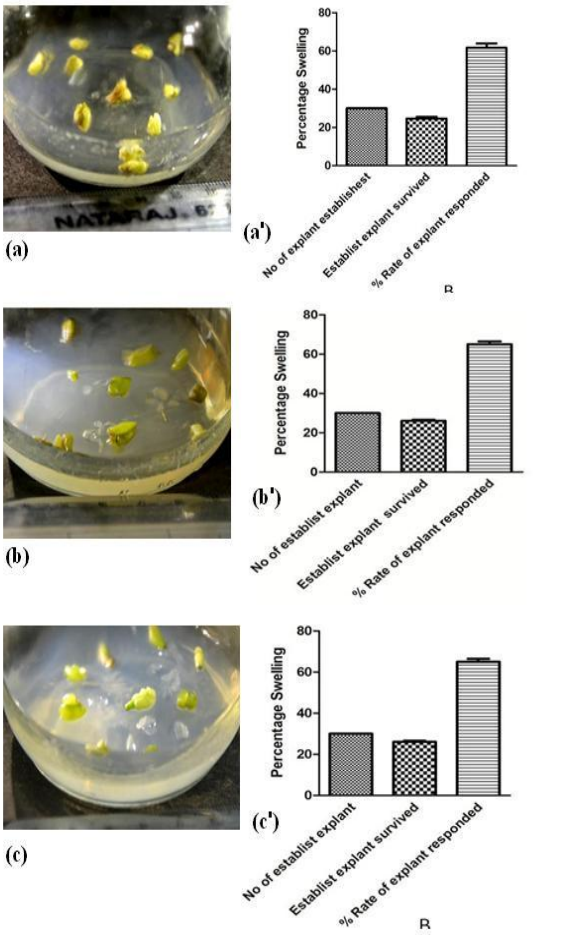

Fig.3: Pictorial and graphical representation of swelling and swelling per cent respectively of *Brassica fruticulosa* X *B.rapa* var. brown sarson observed in medias M1 (a & a'), M2 (b & b') and M3 (c & c ').

C. Swelling percentage of crossed pistil in controlled environmental conditions: Swelling of established pistils was observed under aseptically cultured pistils on the same media M1, M2 & M3. The swelling percentage was observed highest in M3 as compared to M2 and M1 media respectively (Fig.3a-a', b-b' & c-c').The effect of media composition was also reflected in the swelling of cells of established explants. This could be because of intake of water from the medium and onset of internal cell growth of the explants. The observed swelling also could be influenced by both composition of medium and the explants. Initially swelling was localized at one point of explants and then entire portion of the explants, even in some cases entire explants swelled since from beginning. The axially organized growth of cell and tissue irreversibly formed shapeless bulk of living matter under optimum culture conditions.

Callus development: This could be because of intake of water from the medium and onset of internal cell growth of the explants due to axial gradients of bioelectric potentials arising from changes in membrane ionic permeability, primarily to $Ca^{2}+$, K^{+}, Cl^{-}, and H^{+}.The observed swelling also could be the influenced by both composition of medium and the treatments of the 2-4D. The parameters for observation of callogenesis responses regarding growth colour and nature of callus along with the formula of callogenesis percentage is mentioned in Table.1&2 respectively. Some greenish cream to brownish compact callus was observed in M1 media after 19 days of establishment. Moderate growth of callus was observed in M2 media in 19 days characterized with

greenish cream to white having compact with some friable regions. However, the significant callogenesis was observed in M3 media having highest callus induction percentage. The callus was induced in 21 days and was greenish cream with friable regions (fig.4a-a', b-b' & c-c'). Here also, effect of media can be realized clearly. The sub-cultured callus on this media and shoot initiation is depicted in fig.4 d & e respectively. These results were in accordance with the results of Agnihotri *et al.* 1990, Bajaj *et al*, 1986, Chrungu et al 1999, A. 2004; Ji-Peng Chen, 2011; and Atri, C. 2012 on similar basal media and media additives.

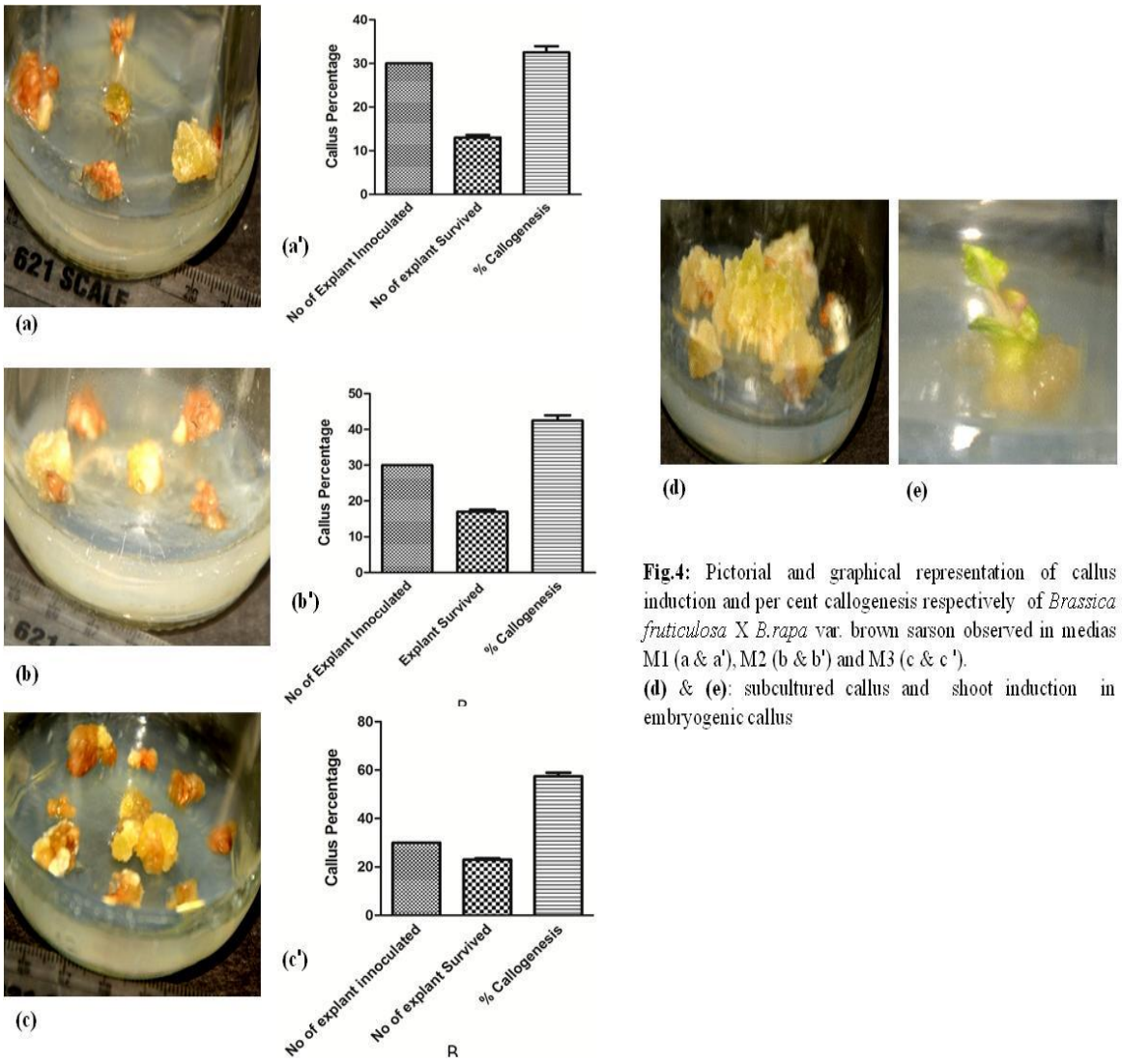

Fig.4: Pictorial and graphical representation of callus induction and per cent callogenesis respectively of *Brassica fruticulosa* X *B.rapa* var. brown sarson observed in medias M1 (a & a'), M2 (b & b') and M3 (c & c '). **(d)** & **(e)**: subcultured callus and shoot induction in embryogenic callus

Table 1: Parametersfor observationof callogenesis

S. No	Symbol	Explanation	Growth measurement		Callogenesis(%)
			Callus in (cm)	Shoot length	No. of explants produced callus ÷ total no. of explants in callusing media **X** 100
1	+	Less growth	**<0.5**	**<0.5**	
2	++	Moderate growth	**1.0-2.0**	**1.0-2.0**	
3	+++	Good growth	**>2.0**	**>2.0**	

Table 2: Growth, colour and nature of callus observed under different media of *B.fruticulosa* X *B. rapa* pistils

S. No	Media name	Growth nature of callus	Colour of callus	Nature of callus	Days taken
1	**M1** (Full MS media+3% sucrose)	+	Greenish cream to brownish	compact	19
2	**M2** (Full MS +Caesine hydrolysate($0.5gl^{-1}$) +3% sucrose)	++	Greenish cream to white	Compact with some friable regions	19
3	**M3** (Full MS media + Caesine hydrolysate ($0.5mgl^{-1}$)+Banzoaminopurine ($0.5mgl^{-1}$) +3% sucrose)	+++	Greenish cream	friable	21

CONCLUSION

Use of 2, 4-Dichlorophenoxyacetic acid in plant tissue culture and its allied agriculture field have also been attempted to increases the growth and developments of explants. Here, we observed longer pod length in 2, 4-Dichlorophenoxyacetic acid treated pistilsin different independent crosses of *B.fruticulosa* and *B. rapa* genetic background, suggesting that there could be some cell dependent common mechanism for longer pod length development. Our result with more thickness of pod length, with increasing size of pod length irrespective of individual crosses, we conclude that 2, 4-Dichlorophenoxyacetic acid is absorbed by the pistil and accumulate in the different cells and can induce growth in independent manner in the cell. Therefore this understanding may have broad implications in plant tissue culture.We also conclude that the observed appearance of callus formation in crosses in *Brassica* species could be analysed to be the best suited for the embryo rescues techniques with respect to present protocol of the embryo rescues with the shoot and root developments. Our result with almost no accumulation of anthocynin in culture media could be indicative of less stress and better for callus developments. Therefore, this optimised protocol of the embryo rescue may be helpful for a breeder to widen the gene pool especially through prebreeding approaches in *Brassicas* and for mapping/tagging of untapped gene (s).

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