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Full Length Research Paper

Isolation of low erucic acid-containing genotype of Indian mustard (*Brassica juncea* Czern. and Coss.) through F_1 hybrid anther culture

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Reciprocal crosses were done between two cultivars; cv. RJ15 and cv. RLM198 of Indian mustard (*Brassica juncea*). Anther derived lines designated as A_1 plants, were raised through anther culture from these F_1 hybrid plants. 45% germination was obtained from distinctly shriveled and small A_1 seeds and grown along with the F_2 plants in the same agro-climatic conditions. Subsequently the lines were compared for inheritance pattern between the lines. A normal frequency distribution curve for siliqua per plant was obtained in all the lines reflecting a similar pattern of recombination. Few seeds from the plants of each lines exhibiting high number of siliqua per plant, were isolated for analysis of erucic acid. Three plants in which erucic acid content was lower than the parent cultivars of A_2 generation were identified. This showed that contrasting characters could be obtained from A_2 plants where the traits are oligo or monogenic through anther culture.

Key words: Anther culture, *Brassica juncea*, Erucic acid, Gas chromatography, Scanning Electron Microscopy (SEM).

INTRODUCTION

Oleiferous Brassicas are major source of edible oil used in several parts of the world. Oil of *Brassica juncea* (Czern. and Coss.), the Indian mustard is consumed in large quantity and the production ranks second among all oilseeds in India (Chopra and Prakash, 1991). But due to the presence of undesirable long chain fatty acids like ecosenoic acid (10%) and erucic acid (50%) in the seed oil, it becomes detrimental to human health. Erucic acid increases blood cholesterol, interferes in myocardial conductance and shortens coagulation time (Renard and Mcgregor, 1992). European economic committee has restricted cultivation of *Brassica* crop that contains more than 10% erucic acid content in their oil (Dhillon et al., 1992). Several works through selection, mutation as well as conventional breeding and modern biotechnological techniques have been reported for developing mustard variety containing low erucic acid (18:2) (Anand and Downey, 1981; Chen et al.,1988). The efforts have been largely focussed to *Brassica napus* with AABB tetraploid genome (Downey and Craig, 1964; Jonsson, 1977), leading to development of '0' erucic acid containing varieties. So, it will require considerable efforts to develop such Indian mustard (*B. juncea*) variety.

Eversince the report of Guha and Maheswari, (1964) about *in vitro* generation of plants with gametic chromosome number through anther culture, haploidy has gradually extended from rice to other crops (Han and Huang, 1987; Rouland et al., 1990). Attempts have been made, using micros-pore/anther culture technique for improving both qualita-tive and quantitative traits of the existing genotypes. The haploids/dihaploids reveals new and beneficial gene combination when compared with conventional hybridiza-tion. In *Brassica*, haploidy resea-rch has advanced to a stage where culture conditions can be defined for any cultivar (Swanson 1990; Prabhudesai and Bhaskaran, 1993). Non–Mendalian segregation ratio was obtained in haploid anther culture derived haploid plant population of. *Nicotiana sp*. (Melcher, 1972). In *B. napus*, haploidy was used to express several recessive

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traits using light seeded Canola strain (Henderson and Pauls, 1992).

Using F_1 hybrid anther as the source for haploid / dihaploid plant production thereby fixing the new product(s) of recombination has been applied in several crops to isolate variants (Henderson and Pauls, 1992). Similarly in the present investigation, anther derived plants (A_1) was raised from anthers of F_1 hybrids plants obtained from crossing two cultivars of *B. juncea*. Subsequently the seeds of A_1 were sown along with F_2 population to study the inheritance pattern of poly and oligo gene governed traits within the lines.

MATERIAL AND METHODS

Material

Seeds of the parental lines, i.e., cv. RLM-198 and cv. RJ-15, were collected from Pulses and Oilseed Research Station, Berhampur, West Bengal, India. The reciprocal hybridization in between the two cultivars [$F_{1/n}$ = RLM198 (♀) x RJ15 (♂) & $F_{1/r}$ = RJ15 (♀) x RLM198 (♂)] was done at Madyamgram Experimental Station of Bose Institute, Calcutta, following the method of Downey and Harvey (1963).

Anther culture technique

Floral buds containing microspore at uninucleate stage, were collected from F_1 hybrid plants and surface sterilized with $HgCl_2$ ((0.5% w/v) using teepol as surfactant for 5 min. The anthers within the floral buds were isolated and separated from the filaments. Sterilization, dissection and inoculation were performed under laminar airflow hood. Modified B*5* medium (Keller et al., 1975) with 12% sucrose supplemented with NAA and BAP was used for anther culture. The *in-vitro* developing microspores were periodically squashed, stained with 2% (w/v) acetocarmine and observed under microscope. Plantlet regeneration and rooting were done according to previous report (Roy and Saha, 1997). After multiplication, the regenerated microspore-derived plantlets were transferred to pots. Seeds were collected from these designated A_1 plants inside the green house. Subsequently, the A_1 seeds were sown along with F_1 seeds in the next Rabi cropping season. Approximately 200 plants were raised in each line and at maturity the number of siliqua in each plant was noted. A frequency distribution curve was prepared and subsequently few plant from each line showing high siliqua per plant, were selected and harvested separately. Erucic acid (ω22:1) was estimated from the seeds of these selected plants individually.

Fatty acid analysis

The seed oil was extracted with n-Hexane in a Soxhlet apparatus for 8 cycles. The oil was dried in vacuum. Triglycerides were separated in 0.5 mm thick silica gel plate (60 – 120 mesh). The spots were identified by standard triglycerides (Sigma Chemicals Co., USA) and scrapped subsequently dissolved in methanol. Furthermore, it was subjected to acid hydrolysis with methanol and H_2SO_4. The fatty acid methyl esters were extracted with diethyl ether (Mishra and Ghosh, 1991). The hydrolyzed product was purified by preparative TLC and analyzed through Gas Chromatography (Hewlett Packard, Series –II) using a diethyl glycol succinate column with flow rate of 60 – 80 ml/min. Sample volume of 2 µl was injected into the column set at a temperature of 380°C. The detector temperature was fixed at 180 – 200°C. Individual fatty acids peaks were obtained through an attached integrator (Hewlett Packard 3394A). The retention time of the peaks were matched with standard fatty acids (Sigma Chemical Co., USA).

Scanning electron microscopy

Anthers with developing callus were fixed, dehydrated and freeze dried with liquid CO_2. They were observed under SEM (Phillips-PSEM500) following to the method of Kott and Kasha (1984).

RESULTS AND DISCUSSION

The inoculated cream-colored anther wall initially turned yellow and then brown in the medium. After squashing these anthers, multicelled microspore with callus forming tendency was observed within 8–10 days. The anther wall ruptured along the line of dehiscence suture (Figure 1a) and yellowish white callus emerged in 25 –30 days. In another 20–25 days, green zones developed at random on the surface of the callus. In the next 30 days, leaves emerged from the surface of the calli (Figure 1b). Androgenic callus induction, regeneration, rooting and transplantation to pots were achieved as described in the previous report (Roy and Saha, 1997).

In all 73 microspore derived plantlets were transplanted from the F_1 anther culture of hybrid lines ($F_{1/n}$ & $F_{1/r}$) and kept in pots covered with polythene bags. The bags were removed after 30 days. At maturity, the plants exhibited a bushy appearance devoid of main stem with several branches emerging from basal portion (Figure 1c). These A_1 lines exhibited different pod size and small seed **(**Figure 1d-e). In several transplanted plantlets though growth and siliqua formation was observed but no seeds were formed. This unconventional plant morphology may be due to the residual effect of *in-vitro* conditions along with hormonal combination, which are added artificially in the medium. Furthermore, the plant being regenerated from tissue other than seed/embryo, which has defined root-shoot meristematic zones, led to this morphology. Moreover, this has not only influenced the plant habit but also led to irregular organ morphology like differential siliqua size, distinctly small and shriveled seeds. The irregular plants habit along with floral morphology of microspore derived transplanted plants have been previously reported (Keller and Amstrong, 1978).

Seeds of the A_1 generation showed 45% germination and 200 plants of each ($F_{2/n}$, $F_{2/r}$ and A_2) line were plotted in the field. On contrary to A_1 generation, the plants of A_2 generation showed normal plant habit, flowering and seed setting. During harvest in A_2 generation, siliqua per plant in population exhibited a normal frequency curve. In most of the A_2 plants, number of siliqua ranged in between 250 to 850 per plant (Figure 2). Highest frequency was found in 251 to 450 classes. As siliqua per plant is influenced by environmental factors therefore it needs to be checked in subsequent generations. Furthermore, from the observation it seems that most of the recombination regarding siliqua per plant is more or less in the si-

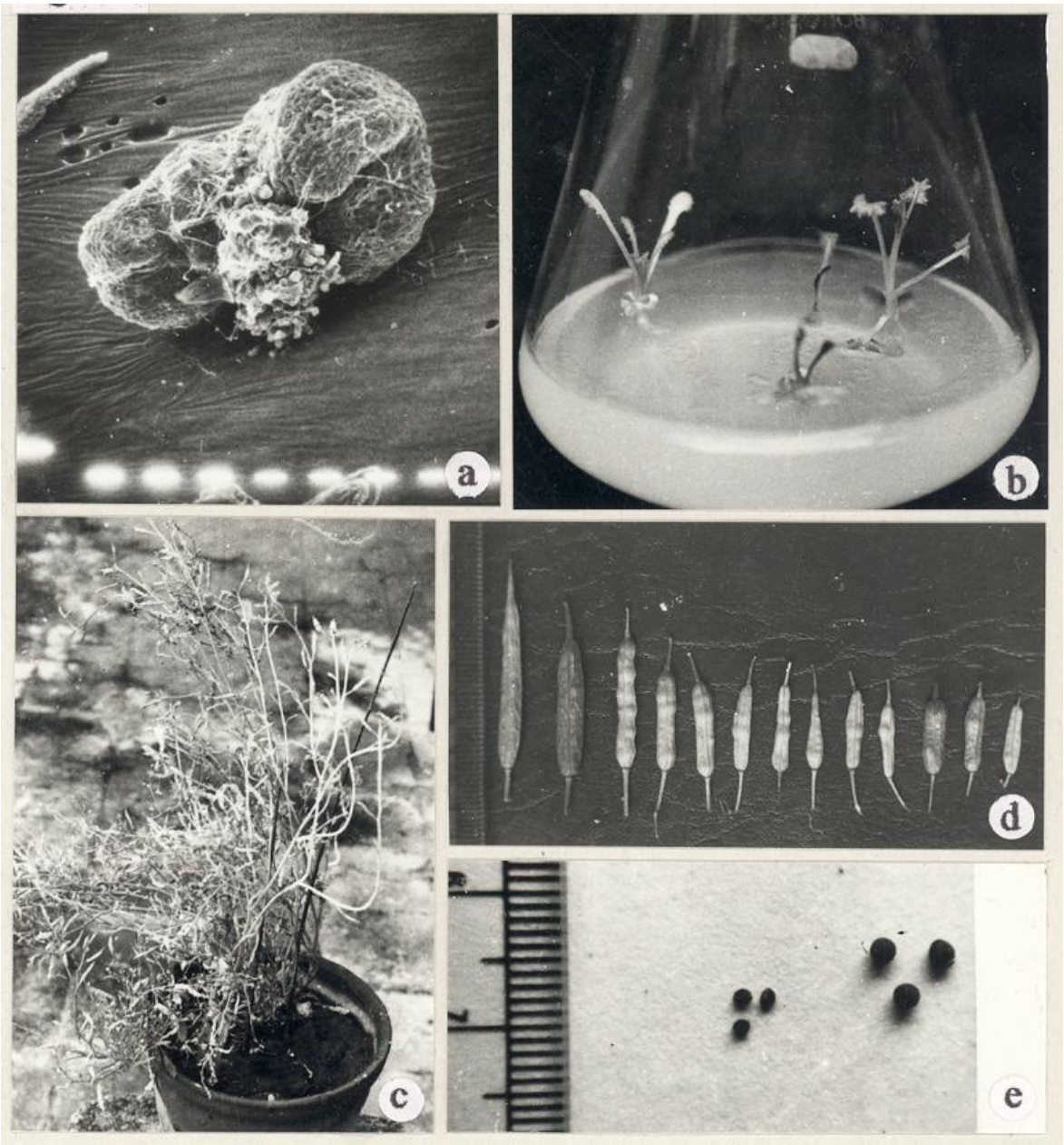

Figure 1. a. Emergence of microcallus along the dehiscence suture, viewed through SEM [Bar represents 100 µm]. **b.** Leaflet regenerated from microspore-derived callus. **c.** Microspore derived A_1 plant transplanted to pots showing habit without a main stem. **d.** Different size of pods of the transplanted A_1 plants [Normal pod of cv. RLM-198, placed in extreme left]. **e.** Different size and shape of seeds obtained from A_1 plants [Three seeds of cv. RLM-198, placed on right side].

milar range and maternal/cytoplasmic inheritance in this trait seems to be negligible. Segregation analysis of isozyme marker on isolated microspore derived embryo of *Brassica napus* showed a similar result (Foisset et al., 1997). Again, to study the inheritance pattern an oligogene-governed trait, erucic acid content among the fatty acid in the seed oil was estimated. It is known that ω24:2 content is governed by two genes with additive effect. Few plants from each of the three lines were selected exhibiting relatively higher siliqua yield per plant.

The erucic acid profile of these selected plant revealed a short narrow range. The erucic acid content in both parents was high and was nearly half of the entire fatty acid present in the seed oil. The brown seeded cultivars RLM-198 showed higher content (52%) while the yellow seeded cultivar RJ-15 (46.2%). The F_2 population of the crosses exhibited erucic acid content in the range of 39.2 % to 49.2%. Out of nine selected genotypes analyzed of the A_2 progeny, $A_{2\text{-}133}$ contained 21% erucic acid in the seed oil (Figure 3). Embryos obtained from microspore

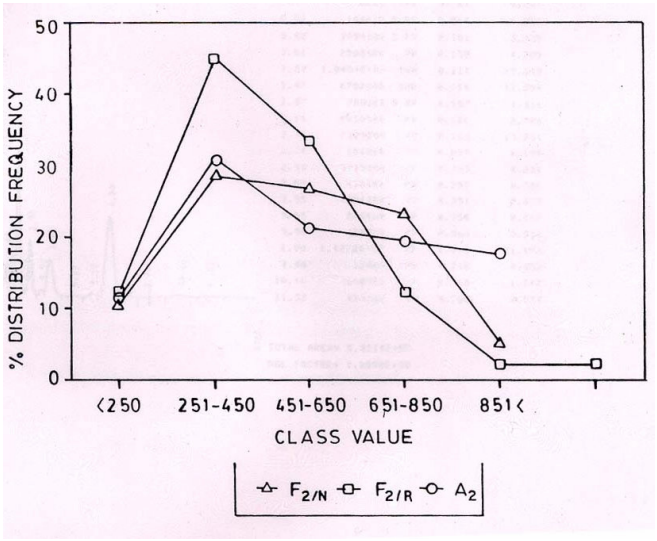

Figure 2. Frequency distribution (%) of pods per plant represented as class value in the F_2 reciprocal hybrid population and microspore derived plant progenies (A_2). [$F_{2/n}$: RLM198 (♀) x RJ15 (♂), $F_{2/r}$: RJ15 (♀) x RLM198 (♂)].

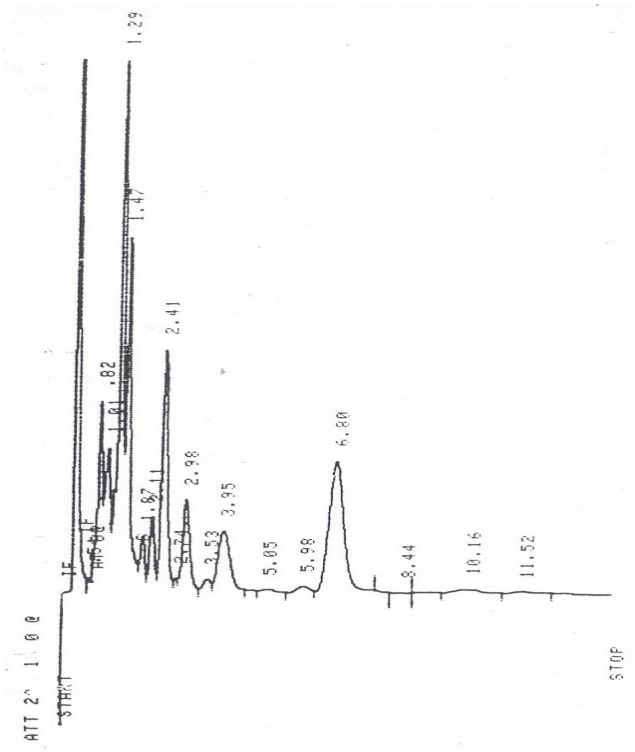

Figure 3. Gas chromatogram of A_2–133 plant showing 21.18 % erucic acid (22:1 ω11) content in seed oil in term of peak area with 6.8 retention time (Rt).

derived *B. campestris* F_1 hybrid seeds showed a wide range of erucic acid content in the popu-lation (Keller et al., 1992). Erucic acid content in seed is regulated by more than one gene (Kirk and Hurlstone, 1983) and in *B. juncea,* two genes acting in additive fashion have been implicated. Therefore, due to recombi-nation in between the parental alleles and in the dihaploid genome due to the absence of allelic pair, the erucic acid content in A_2 population has varied and particularly decreased in few plants. On contrary the yield traits being multigene governed, the recombination and subsequent phenotypic influence remained similar in A_2 plants when compared with that of F_2 plants. It would be interesting to compare single gene governed traits as drastic recombi-nants can be expected within these three lines. Also the status of erucic acid content in $A_{2\text{-}133}$ needs to be verified along with the yield trait in the subsequent generations, as it would be agronomically useful.

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