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RESEARCH ARTICLE

Anther androgenesis in response to culture media and donor genotypes in brinjal (*Solanum melongena* L.)

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Abstract

Androgenesis offers a rapid approach for development of completely homozygous lines, however its efficiency depends upon donor genotype and culture conditions. This study aimed to optimize the androgenesis using different donor hybrids and Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of growth regulators for callus induction and regeneration in brinjal. Among different media combinations, MS medium supplemented with 2 mg/l IAA and 0.5 mg/l Kinetin developed the highest and earliest calli (65.8% in 28.5 days). Among donor hybrids, SC-15-2 x MR-319 produced the maximum (73.4%) calli in 13.5 days. Following subcultures of induced calli, MS supplemented with 0.2 mg/l IAA and 4 mg/l Zeatin achieved the highest regeneration (19.5%) in 21.4 days. Among donors, SR-5 x SR-9322 revealed 15.5% calli regeneration in 21.7 days. This donor hybrid also demonstrated direct regeneration from cultured embryos in MS + 2 mg/l IAA + 0.5 mg/l Kinetin medium which turned into shoots and developed roots upon sub-culturing. In addition, ½ MS added with 0.5 mg/l IBA induced rooting in 58.3% of rootless cut-shoots that were hardened to complete plants following *in-vitro* and *ex-vitro* methods for acclimatization. An SSR marker emf21M05 confirmed gametic origin of 20 regenerants. Cytological analysis of metaphase-1 stages in dividing pollen mother cells of 24 regenerants categorized the plants into diploid (4), haploids (9), doubled-haploids (11) for further crop improvement in brinjal.

Keywords: *Solanum melongena* L., Androgenesis efficiency, Growth regulators, Double haploids, SSR validation.

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Introduction

Brinjal (*Solanum melongena* L., 2n = 24) is an often cross-pollinated crop that largely relies on conventional breeding approaches for genetic improvement with respect to nutritional quality, stress tolerance, and yield potential. Its substantial genetic variability, encompassing diverse fruit types, offers considerable scope for crop enhancement (Kalloo 1993). Despite this variability, heterosis breeding depends on the availability of uniform and stable inbred lines. The development of such inbreds through traditional selfing requires 6 to 7 generations and is constrained by limitations of time, land, and labor.

Doubled haploid (DH) technology provides a rapid alternative for achieving complete homozygosity within 1 to 2 years *via* androgenesis. Among the various approaches for haploid production, androgenesis, gynogenesis, and irradiated pollen techniques have been widely explored (Murovec and Bohanec 2012). In androgenesis, uninucleate microspores within cultured anthers are induced to form haploid plants, which can subsequently be converted into doubled haploids through chromosome doubling. These DH lines are highly valuable for genetic studies, including gene

transfer, detection of recessive mutations, linkage mapping, marker–trait association, and gene tagging (Sahana *et al*. 2024).

Although androgenic response in brinjal was first reported by Raina and Iyer (1973), subsequent studies by Isouard *et al*. (1979) and the Chinese Research Group of Haploid Breeding (1978) successfully achieved haploid production. The efficiency of androgenesis is influenced by several factors, including genotype, season of donor plant growth, developmental stage of the anther, flower bud maturity, explant pre-treatments, culture media composition, and *in-vitro* incubation conditions. About 4 hours temperature pre-treatment (33–35°C) to anthers for microspore culture has been shown to enhance androgenic response and induce spontaneous chromosome doubling in uninucleate microspores that notably produced relatively high frequency (up to 69.3%) of spontaneous doubled haploid formation in both anther and microspore cultures (Rivas-Sendra *et al*. 2015).

However, haploid plants are generally weak, small, and sterile, necessitating artificial chromosome doubling. Colchicine, a widely used antimitotic agent, disrupts microtubule formation by binding to tubulin, resulting in chromosome doubling efficiencies of 50 to 70% (Gemesne Juhasz *et al*. 2001). Reliable determination of ploidy levels in regenerated plants can be achieved through cytological analysis and flow cytometry, while the gametic (homozygous) or somatic (heterozygous) origin of regenerants can be confirmed using co-dominant SSR markers (Murovec and Bohanec 2012). Despite previous reports of successful androgenesis, complete information of regeneration efficiency in different fruit group donors and their validation through SSR markers and cytology was insufficient. Therefore, the present study was undertaken to evaluate the influence of donor genotype and culture media on anther androgenesis in brinjal.

Materials and Methods

Plant material and explant preparation

The experimental material comprised of eight hybrid combinations derived from stable germplasm lines representing diverse fruit groups, maintained at the Department of Vegetable Science. Immature, healthy flower buds were excised from fully grown donor plants, placed in an ice box, and transported to G.S. Khush Laboratories, School of Agricultural Biotechnology, PAU, Ludhiana. The buds were identified for early- to late-uninucleate microspore stage, corresponding to 4.5 mm greenish-yellow anthers as shown in Figure 1A & B (Ellialtoglu *et al.* 2012). Aseptic conditions were maintained by UV-sterilizing the laminar airflow cabinet for 15 minutes, followed by air drying in the laminar air flow for 30 minutes. All culture accessories were surface-sterilized using 70% ethanol. The excised buds were pre-cooled at 4°C for 3 hours, rinsed with autoclaved distilled water, and surface-sterilized with 70% ethanol for 30 seconds. Subsequently, they were treated with 10% (10 mL/100mL solution) commercial Rin Ala Fabric Whitener (4% Sodium hypochlorite, 1% sodium hydroxide and 1% amine oxide) for 15 minutes under aseptic conditions (Figure 1C) and rinsed three times with sterile distilled water to remove residual disinfectant.

Callusing and regeneration

Anthers were aseptically excised from surface-sterilized buds using sterile forceps and scalpel (Figure 1D), and cultured in jam jars (110 × 65 mm) containing pre-poured MS medium (Murashige and Skoog 1962) containing 20% sucrose, solidified with 0.8% agar, and maintained at 5.8 pH and supplemented with different concentrations and combinations of plant growth regulators for callus induction. For each treatment, three replicates were maintained, each consisting of 12 culture jars containing 15 anthers per jar. The inoculated culture vessels were sealed with parafilm and incubated at 35°C in dark for 8 days to initiate cell division in microspores (Basay and Ellialtıoglu 2013), followed by callusing at 25 ± 2°C in the dark.

The induced embryogenic calli were then transferred to a solid MS medium supplemented with various concentrations and combinations of BAP (0.5–5 mg/l), Kinetin (0.5–5 mg/l), IAA (0.2–4 mg/l), and Zeatin (0.2–4 mg/l) along with 20 g/l sucrose. Cultures were incubated at 25 ± 2°C under a 16/8 h light/dark photoperiod with a light intensity of 4000 lux (80 micromoles m^{-2} sec^{-1}), with subculturing at 3-week intervals until complete plantlet regeneration. Directly regenerated embryos emerged from expanded anthers were also repeatedly cultured on same medium for plantlet formation. Unrooted shoots were excised and transferred to half-strength MS medium supplemented with 0.5 mg/l IBA for root induction.

Rooted plantlets were carefully removed, washed to eliminate adhering medium, and placed on moist sterile cotton for hardening for one week. Moisture was maintained by daily application of sterile distilled water. Hardened plantlets were subsequently transferred to black polythene bags (12 × 7.5 cm) containing sterilized substrate (cocopeat: FYM: perlite: vermiculite in 2:2:1:1 ratio) and maintained at 25°C, 70 to 80% RH and 16/8 h light/dark photoperiod for acclimatization. The observations were recorded for anther expansion, callus formation, direct, callus regeneration, and plantlet survival.

Confirmation of genetic origin

For the confirmation of the genetic origin, one SSR markers viz; emf21M05 (FP-5'- CTCCCAAGACCTGGAAGTCACCTA-3' and RP-5'-AGAAGCCTTGCCACTTGGCTTAAC-3') polymorphic to the donor hybrid was used to ascertain the presence of maternal/paternal alleles only. The genomic DNA of the

parents, donor and regenerated plants was extracted in triplicates from young growing leaves (1g approx.) as per the CTAB method Doyle and Doyle (1990). Each DNA sample received 5 µL of 40 U/µl RNase treatment and one-hour incubation in a water bath set at 37°C. A Nano-DropTM 1000 spectrophotometer (Thermo Scientific) was used to examine the purity and quantity of isolated DNA samples. DNA dilution for each sample @50 ng/mL was prepared for polymerase chain reaction (PCR). PCR was performed with a 12.4 µL reaction mixture containing 6 µL of master mix (Takara, Japan), 0.7 µL of MgCl2 (25 mM), 1.5 µL of forward and reverse primer (5µM), 1.8 µl of nuclease-free water, and 1.5 µL of total genomic DNA (50 ng/µL). In an Eppendorf thermocycler, PCR underwent four steps. Initial denaturation took place in stage 1 for 3 minutes at 95°C. In stage 2, twenty additional cycles of denaturation at 95°C for 45 seconds were followed by 45 seconds of primer annealing at 58°C (-0.5°C/cycle) and 45 seconds of DNA chain extension at 72°C. In stage 3, 10 cycles of denaturation at 95°C for 45 seconds were followed by the primer's annealing at 50°C for 45 seconds and the extension of the DNA chain at 72 °C for 45 seconds. Stage 4 involved a final extension at 72°C for 7 minutes and a hold at 4°C for an infinite time. The amplified products were resolved at 100 volts for 5 to 6 hours on a 3% agarose gel using 0.05 µL/mL ethidium bromide. The amplified bands were visualized under UV illumination and pictures were taken using the Gel Documentation System (Alpha Imager HP, USA). Plants that amplified only a single band (200–300 bp) parallel to either of the parents were considered to have originated from haploid microspores. Plants that amplified two bands parallel to the heterozygous donor were considered to have appeared from somatic origin (Figure 2).

Cytological analysis was necessary to distinguish the ploidy status of haploid and doubled-haploid regenerants. To perform this analysis, immature flower buds (10 mm) with actively dividing pollen mother cells were excised from the regenerant at the reproductive stage and subjected to cytological examination. The cell division in pollen mother cells from the regenerants and diploid donor was fixed separately in 40 mL of Carnoy's fixative (ethanol: chloroform: acetic acid in a ratio of 6:3:1) for 24 hours, then transferred to 70% alcohol and kept at 4°C until their better visualization. To visualize, the anthers of fixed buds were removed and crushed in 2% acetocarmine. Pollen mother cells showing diakinesis or M-I (metaphase-I) and anaphase-1 stages of cell division were focused and photographed for counting the chromosome numbers. Several meiotic stages from each slide were analyzed in several pollen mother cells for selected plantlets. Based on the chromosome count at metaphase-1 and succeeding stages (Anaphase-1), the ploidies of different regenerants cells in five slides for each were ascertained in comparison to their diploid donors.

Statistical analysis

Factorial CRD design (Fisher 1935) was used to conduct statistical analysis of variance from replicated data of media (first factor) and genotype (second factor) for various callusing and regeneration parameters, and Tukey's test was applied to compare the means with $p \leq 0.05$ using SPSS statistical software (SPSS, 2008).

Results and Discussion

Anther callogenesis in response to media combinations

In the present study, only media that produced non-watery, creamy-white, compact-embryonic calli and later regenerated into plantlets are presented in Table 1 & Figure 1E). Excluding the effect of donor hybrids, anther expansion on selected media combinations the varied between 49.31 and 68.47%. The MS medium supplemented with 2 mg/l IAA and 0.5 mg/l Kinetin produced 65.8% calli, which was statistically comparable to MS + 2 mg/l NAA + 1 mg/l BAP (65.5%), and greater than MS + 2 mg/l NAA + 2 mg/l BAP (58.1%). However, MS + 2 mg/l 2, 4-D + 1 mg/l BAP, being at lowest level, induced 55.4% calli. MS fortified with 2 mg/l IAA and 0.5 mg/l Kinetin formed the earliest calli (28.5 days) in comparison to other media combinations. While the callus initiation times of MS + 2 mg/l NAA + 1 mg/l BAP and 2 mg/l NAA + 2 mg/l BAP were statistically similar with 29.6 and 30.1 days, respectively. It took the longest (33.9 days) for calli to initiate in MS medium added with 2 mg/l 2,4-D, and 1 mg/l BAP. On an average, only MS supplemented with 2 mg/l IAA and 0.5 mg/l Kinetin demonstrated direct embryogenesis from anthers (2.6%) (Figure 1G).

Among the 20 media combinations evaluated for standardization, MS medium supplemented with 2,4-D, IAA, NAA, BAP, and Kinetin (0.5–2 mg/ l in various combinations)

Table 1: Anther callogenesis in response to different growth hormones in brinjal

Media combination	*Anther expansion (%)*	*%Callusing*	*Days taken to calli initiation*	*Direct embryo regeneration (%)*
MS + 2 mg/l IAA + 0.5 mg/l Kin	68.47[a]	65.8[a]	28.5 ± 7.1[c]	2.6[a]
MS + 2 mg/l NAA + 1 mg/l BAP	55.6[c]	65.5[a]	29.6 ± 14.4[b]	-
MS + 2 mg/l 2,4-D + 1 mg/l BAP	49.31[d]	55.4[c]	33.9 ± 12.4[a]	-
MS + 2 mg/l NAA + 2 mg/l BAP	60.97[b]	58.1[b]	30.1 ± 14.1[b]	-

Means are separated using a Tukey's test ($p \leq 0.05$).

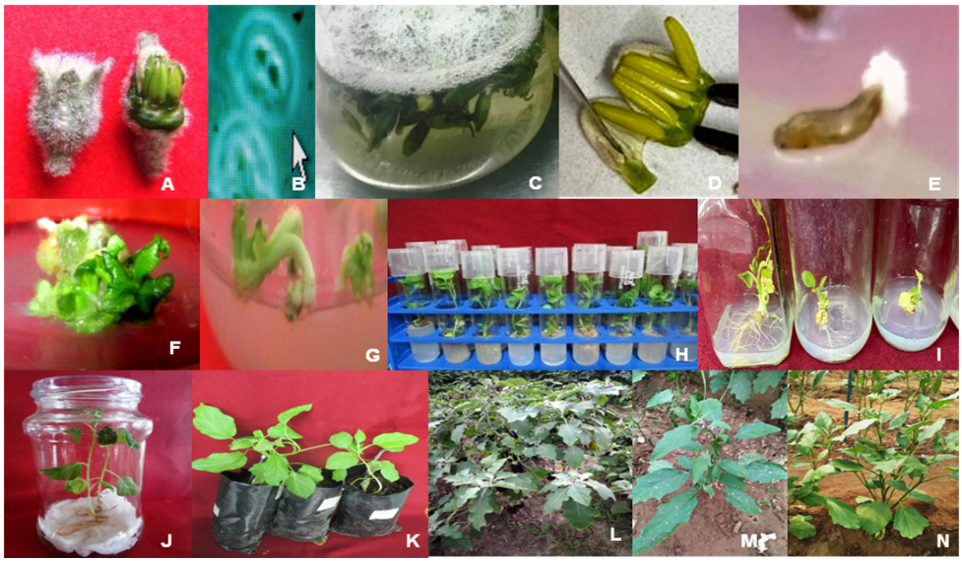

Figure 1: Anther anderogenesis in brinjal: A. flower buds, B) early binucleate stage of pollen, C) bud sterilization, D) greenish-yellow anther excision, E) calli popping out of anther, F) calli regeneration, G) direct embryo regeneration, H) regenerated plantlets, I) root induction in half strength MS medium, J) *In-vitro* hardening on moist cotton, K) hardening of plantlets in polythene bags, L) F_1 donor, M) haploid plant, N) doubled haploid plants

supported effective callus induction and embryogenesis. Auxins promoted cell division and dedifferentiation of anther wall tissues, while microspores responded rapidly, leading to anther swelling and, in some cases, rupture. However, higher concentrations of the strong auxin 2,4-D resulted in excessive, friable, and watery callus that lacked regenerative potential. Improved callus quality was observed when weaker auxins such as IAA and NAA were combined with cytokinins (BAP or kinetin) at moderate levels (0.5–1 mg/l). These combinations facilitated the formation of compact, embryogenic callus, typically emerging either from the tip or through the rupture of the anther. A balanced auxin–cytokinin ratio appeared critical in directing the developmental pathway of callus toward embryogenesis and subsequent plant regeneration in literature (Sahana *et al.* 2024). The role of auxins in initiating cell division in microspores was evident, with 2,4-D showing the strongest effect, whereas IAA and NAA were more favorable for producing regenerable callus when used in combination with cytokinins. To improve direct regeneration, which can transform microspores to embryoids just after their initial divisions, our results suggested that initially expanded anthers be transferred to a medium with an increased level of cytokinins ranging from 0.5 and 1.0 mg/l.

Anther callogenesis in response to donor

The response of different donor hybrids for anther expansion, callogenesis, and embryogenesis are summarized in Table 2. Anther expansion in different donors was observed in a range of 32.7-80.8%. Further, expanded anthers from SC-15-2 × MR-319 could induce 73.4% calli which was statistically similar to 71.3% calli induced in SR-5 × SR-9322. SVL-359 × BLW-231 and SR-9322 × W-230 responded moderately, whereas PSB-7-2 × P-219 showed the lowest

Table 2: Anther callogenesis in response to different donor hybrids in brinjal

Donor hybrids	*Expanded anthers (%)*	*%Callus induction*	*Days taken to calli initiation*	*Direct regeneration (%)*
SC-15-2 x MR-319	79.7^{b}	73.4^{a}	13.5±2.4^{f}	-
SVL-359 x SR-9322	40.2^{f}	56.1^{d}	41.8±6.8^{b}	-
SVL-359 x BLW-231	71.1^{c}	61.2^{b}	24.7±8.5^{d}	-
SR-9322 x W-230	56.8^{e}	60.4^{bc}	37.3±2.2^{c}	-
V-230 x BRG-111	38.3^{g}	57.8^{cd}	37.3±5.9^{c}	-
PSB-7-2 x P-219	69.6^{d}	51.5^{e}	24.7±8.0^{d}	-
W-230-42 x WOB-406	32.7^{h}	58.4^{bc}	44.5±8.4^{a}	-
SR-5 x SR-9322	80.8^{a}	71.3^{a}	20.5±2.9^{e}	4.5^{a}

* Means are separated using a Tukey's test ($p \leq 0.05$).

Table 3: Regeneration response of anther tip calli using different growth hormones in brinjal

Media composition	*% Calli regeneration*	*Days taken to calli regeneration*
MS + 2 mg/l BAP + 0.5 mg/l Kinetin	9.8[d]	29.8 ± 3.4[d]
MS + 0.2 mg/l IAA + 4 mg/l Zeatin	19.5[a]	21.4 ± 2.7[e]
MS + 2.5 mg/l Kinetin + 0.5 mg/l BAP	11.3[c]	34.4 ± 9.1[b]
MS + 2 mg/l Kinetin + 0.5 mg/l BAP	17.0[b]	34 ± 8.1[b]
MS + 3 mg/l Kinetin + 0.5 mg/l BAP	11.2[c]	32.1 ± 9.4[c]
MS + 3.5 mg/l Kinetin + 0.5 mg/l BAP	3.2[e]	36.5 ± 8.3[a]

* Means are separated using a Tukey's test ($p \leq 0.05$).

callusing ability. In terms of callus initiation, SC-15-2 × MR-319 exhibited the fastest response (13.5 days), followed by SR-5 × SR-9322 (20.5 days), while SVL-359 × BLW-231 and PSB-7-2 × P-219 required relatively longer durations. In contrast, W-230-42 × WOB-406 required the longest duration (44.5 days) for callus initiation. Notably, SR-5 × SR-9322 also exhibited 4.5% direct embryogenesis from expanded anthers on callus induction medium (Figure 1G).

The observed variation in callus formation and embryogenesis among donor hybrids in the present study may be attributed to differences in intrinsic auxin levels (Corral-Martinez and Segui-Simarro 2012). Microspores (gametic cells) appeared to respond variably to callogenesis under both higher and lower concentrations of exogenously applied growth regulators. Differential endogenous hormone levels in different donors and their calli might have influenced dedifferentiation, acquisition of embryogenic competence and subsequent embryo development, and to some extent, direct embryogenesis as reported earlier (Caeiro *et al.* 2022). Following *in-vitro* culture, these donors exhibited the highest percentages of anther expansion and callus formation, likely due to an optimal auxin–cytokinin ratio. The differential response among donor genotypes observed in this study is consistent for the findings of significant influence of donor genotype on androgenic development (Sun *et al.* 2023; Sahana *et al.* 2024).

Calli regeneration response to media combinations

In the present study, MS medium supplemented with different combinations and concentrations of growth regulators significantly influenced callus regeneration. Only creamy-white to brownish, embryogenic calli emerging or protruding from tip of cultured anthers were the most suitable for further regeneration. Across the variable regeneration response of media combinations (Table 3 & Figure 1F), MS medium supplemented with 0.2 mg/l IAA and 4 mg/l zeatin presented the highest regeneration frequency (19.5%), followed by MS + 2 mg/l Kinetin + 0.5 mg/ l BAP (17.0%). MS + 2.5 mg/l kinetin + 0.5 mg/l BAP and MS + 3 mg/l kinetin + 0.5 mg/l BAP recorded the statistically similar regeneration rates (11.3 and 11.2%, respectively). MS + 3.5 mg/l kinetin + 0.5 mg/l BAP displayed the poorest response (3.2%) for calli regeneration. In terms of regeneration time, MS + 0.2 mg/l IAA + 4 mg/l zeatin revealed the earliest response (21.4 days), followed by MS + 2 mg/l BAP + 0.5 mg/l kinetin (29.8 days), whereas MS + 3.5 mg/l Kinetin + 0.5 mg/l BAP recorded the longest duration (36.5 days).

Callus regeneration was influenced by the physiological state of the callus, endogenous hormone levels, and the balance of exogenously applied growth regulators. The results suggest that a balanced auxin–cytokinin ratio is critical for embryoid formation and subsequent differentiation (Caeiro *et al*. 2022). Regeneration was most effectively induced at lower auxin concentrations (0.2–0.5 mg/l) in combination with moderate cytokinin levels (2.0–2.5 mg/l). This balance likely facilitated coordinated cell division and differentiation during plant regeneration. An increase in cytokinin concentration up to an optimal level enhanced regeneration efficiency; however, further increases resulted in a marked decline. This reduction may be attributed to hormonal imbalance, where elevated cytokinin levels without adequate auxin limited cell division prior to differentiation. Notably, higher zeatin levels in combination with low IAA enhanced shoot regeneration, indicating its specific promotive role in morphogenesis through cell division and differentiation. Conversely, excessive cytokinin concentrations alone inhibited regeneration, especially elongation of embryos into plantlets, thus suppressing shoot growth. Further, an optimized hormone concentration, particularly by increasing zeatin (up to ~6 mg/l) in conjunction with a proportional increase in auxin (0.5–1.0 mg/l) can potentially improve regeneration efficiency.

Calli regeneration response of donor hybrids

As presented in Table 4 and Figure 1F, the donor hybrids exhibited significant variation in callus regeneration response irrespective of media combinations. Among the hybrids, SR-5 × SR-9322 recorded the highest callus regeneration (15.5%), followed by SC-15-2 × MR-319 (14.3%). The hybrids PSB-7-2 × P-219 (13.6%) and SR-9322 × W-230 (13.1%) were statistically at par. In contrast, V-230 × BRG-111 exhibited the lowest regeneration response (7.5%). The time

Table 4: Regeneration response of anther tip calli of different donor hybrids in brinjal

Donor hybrids	*% Calli regeneration*	*Days taken to calli regeneration*
SC-15-2 x MR-319	14.3[b]	25.6 ± 4.2[g]
SVL-359 x SR-9322	11.7[d]	33.2 ± 5.4[d]
SVL-359 x BLW-231	11.7[d]	34.1 ± 5.4[c]
SR-9322- x W-230	13.1[c]	26.8 ± 3.7[f]
V-230 x BRG-111	7.5[f]	41.7 ± 11.4[a]
PSB-7-2 x P-219	13.6[c]	29.8 ± 5.3[e]
W-230-42 x WOB-406	8.7[e]	37.8 ± 7.7[b]
SR-5 x SR-9322	15.5[a]	21.7 ± 3.0[h]

Means are separated using a Tukey's test ($p \leq 0.05$).

required for callus regeneration also varied significantly among the anther donors. Calli derived from SR-5 × SR-9322 differentiated into plantlets earliest (21.7 days), followed by SC-15-2 × MR-319 (25.6 days), SR-9322 × W-230 (26.8 days), and PSB-7-2 × P-219 (29.8 days). In contrast, V-230 × BRG-111 required the longest duration (41.7 days) for callus regeneration. In the present study, the hybrid SR-5 × SR-9322 also exhibited direct regeneration through anther bursting when cultured on MS medium supplemented with 2 mg/ l IAA and 0.5 mg/ l kinetin. Repeated subculturing at 15-day intervals on the same medium promoted simultaneous shoot development and root initiation in 41.6% plantlets (Figure 1H). Further, shifting of unrooted shoots onto half-strength MS medium supplemented with 0.5 mg/ l IBA and 8 g/l agar induced rooting in 58.4% of the plantlets (Figure 1I). Following one week of *in-vitro* hardening on moist cotton in jam jars, 57 regenerated plantlets (73%) survived (Figure 1J). Subsequently, 40 plantlets (70%) were successfully acclimatized at 25°C for 15 days in black polythene bags (12 × 7.5 cm) containing a substrate mixture of cocopeat, FYM, perlite, and vermiculite (2:2:1:1) with regular watering (Figure 1K), but 24 plants acclimatize on transplanting in soil in net house during spring summer season and reached reproductive maturity under protected conditions (Figure 1L–N).

The variation in regeneration response among donor hybrids may be attributed to differences in callus quality arising from genotype-specific hormonal balance (Sahana *et al*. 2024). Genotypes exhibiting higher regeneration efficiency may possess an optimal endogenous ratio of auxins and cytokinins conducive to enhanced cell division and differentiation. The low survival of anther-derived plants might be due to weak root systems, altered physiological characteristics developed under *in-vitro* conditions, and difficulties in acclimatization (Hazarika 2003). Additionally, genetic and ploidy abnormalities associated with callus-mediated regeneration may reduce plant vigour and field establishment.

Confirmation of genetic origin

During *in-vitro* culture, some regenerants exhibited slow growth which were characterized by smaller, thinner leaves and shorter stems at full grown stage indicating haploid origin (Figure 1M), while showing normal development to complete fertile plants indicated diploid or doubled haploid origin. Notably, the plants with week morphology showed complete sterility at reproductive stage and other plants showed complete fertility (Figure 1N) as also reported earlier (Winarto *et al.* 2011). In the present study, the regenerated plants were considered to be of possible gametic origin because the calli originated from the tips of swollen anthers, suggesting their derivation from microspores rather than somatic tissues. The presence of proembryos beneath swollen and ruptured anthers further confirmed the microspore-derived nature of directly regenerated plantlets and was consistent with the findings of Dissanayake et al. (2020) in cassava.

A polymorphic SSR marker emf21M05 for the donor hybrid (SR-5 × SR-9322) further distinguished microspore origin (gametic origin) of 20 regenerated plantlets from 4 somatic regenerants (Figure 2). This marker exhibited heterozygosity in the donor hybrid and somatic regenerants corresponding to polymorphism between the parental lines (P_1 = SR-5 and P_2 = SR-9322). Due to the diploid nature of brinjal, the heterozygous F_1 hybrid and somatic regenerants amplified a maximum of three polymorphic alleles inherited from the two parents. However, distinguishable mono or di-allelic plants parallel to either parent were assumed to have gametic origins and homozygosity for said allele as per earlier molecular evidence for confirming haploids and diploids in cassava (Dissanayake *et al.* 2020).

In further cytological confirmation, the meiotic studies of anther-derived regenerants at metaphase-1 stage in pollen mother cells at reproductive stage further revealed a chromosome configuration of heterozygous regenerants as diploid (2n = 12 bivalents), homozygous as true doubled haploids (2n = 12 bivalents), and haploids (n = 12 univalents) in comparison to diploid donor parent (12 bivalents) as shown in Figure 3A-C. Therefore, among 24 plants established from anther culture of F_1 donor SR-5 x SR-9322, 4 (16.7%), 9 (37.5%) and 11 (45.8%) were diploid, haploids and, doubled-haploids, respectively. Among doubled haploids, cytological examination of pollen mother cells at early metaphase and anaphase stages revealed the presence of 12 bivalents, and at late metaphase stage showed 24 separating chromatids, indicating homologous chromosome pairing and normal cell division in double-haploids. The likely appearance of univalent formation at late metaphase -1 may be attributed to desynapsis (early pairing followed by chromosome separation) (Figure 3 C) which have been reported in tissue culture-derived regenerants, but plant remains fertile due to balanced segregation and normal division at anaphase-1 (Figure 3D). However, these plants

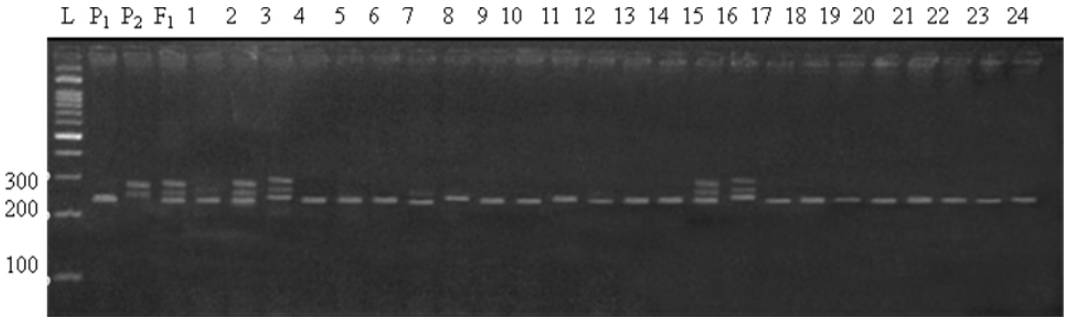

Figure 2: SSR confirmation (emf21M05) of gametic origin of anther-derived regenerants from SR-5 x SR-9322 showing polymorphism among parents (P1 = SR-5, P2 = SR-9322), segregation in anther derived regenerants

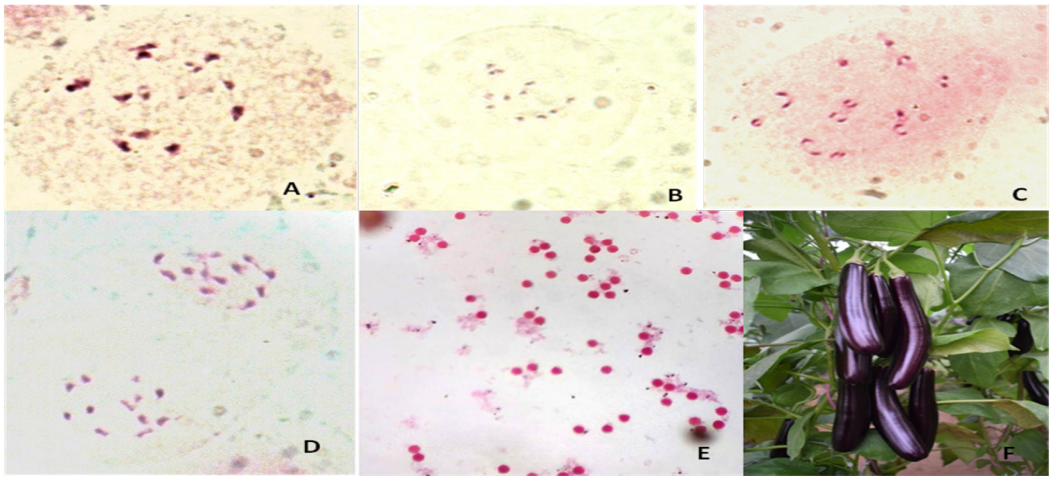

Figure 3: Cytological confirmation of ploidy of regenerants: A) A PMC at metaphase-I showing 12 bivalents (diploid donor) B) A PMC at metaphase-I showing 12 univalents (haploid), C) A PMC at metaphase-I showing 12 bivalents (doubled-haploid), D) Anaphase -1 (12:12 normal separation), E) pollen fertility and F) fruit set ability of in doubled haploid

produced fully fertile pollen and set fruits (Figure 3E and F), indicating normal cell division. Haploid plants had thin long buds with complete sterility. Our results of spontaneous development of doubled-haploids were substantiated with the findings of Rivas-Sendra et al. (2015), and Vural and Ari (2020) in brinjal.

Conclusion

In the present study, immature anthers at the uninucleate to early binucleate stage yielded regenerants of confirmed gametic origin. Genotype and growth regulators significantly influenced callus induction and plant regeneration. Among the tested media, MS supplemented with 0.2 mg/l IAA and 4 mg/l zeatin achieved 19.5% regeneration efficiency that can be attributed to a balanced auxin and cytokinins ratio essential for cell division and cell differentiation, respectively. Additionally, MS medium supplemented with 2 mg/l IAA and 0.5 mg/l kinetin induced direct embryo regeneration in burst anthers, indicating an alternative regeneration pathway. Fine-tuning the balance and proportion of auxins and cytokinins to optimize both callus-mediated and direct regeneration responses can improve androgenesis in future. Overall, genotype-specific responses and precise hormonal regulation is required for enhancing androgenic regeneration efficiency in brinjal. Molecular confirmation of genetic origin along with PMC cytology is necessary to ascertain true doubled-haploids for crop improvement.

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सारांश

एंड्रोजेनेसिस पूरी तरह से समरूप जीनोटाइप के विकास के लिए एक तीव्र दृष्टिकोण प्रदान करता है, हालांकि इसकी दक्षता दाता जीनोटाइप और संस्कृति स्थितियों पर निर्भर करती है। इस अध्ययन का उद्देश्य बैंगन में कैलस गठन और पुनर्जनन के लिए विकास नियामकों के विभिन्न सांद्रता और संयोजनों के साथ पूरक विभिन्न डोनर हाइब्रिड और मुराशिगे और स्कूग (एमएस) कल्चर मीडियम का उपयोग करके एंड्रोजेनेसिस को अनुकूलित करना था। विभिन्न मीडिया संयोजनों के बीच, 2 मिलीग्राम/लीटर आई ए ए और 0.5 मिलीग्राम/लीटर किनेटिन के साथ पूरक एम एस माध्यम ने उच्चतम और सबसे प्रारंभिक कैलआई विकसित की (28.5 दिनों में 65.8%)। डोनर हाइब्रिड में, SC-15-2 x MR-319 ने 13.5 दिनों में अधिकतम (73.4%) कैलआई का उत्पादन किया। प्रेरित कैलआई के उपसंस्कृतियों के बाद, एम एस को 0.2 मिलीग्राम/लीटर आईएए और 4 मिलीग्राम/लीटर ज़ेआटिन के साथ पूरक करने पर 21.4 दिनों में उच्चतम पुनर्जनन (19.5%) प्राप्त हुआ। दाताओं के बीच, SR-5 x SR-9322 ने 21.7 दिनों में 15.5% कैलआई पुनर्जनन का खुलासा किया। इस डोनर हाइब्रिड ने एम एस 2 मिलीग्राम/लीटर आई ए ए 0.5 मिलीग्राम/लीटर किनेटिन माध्यम में संवर्धित भ्रूणों से प्रत्यक्ष पुनर्जनन का भी प्रदर्शन किया जो उप-संवर्धन पर अंकुरों में बदल गया और जड़ें विकसित हुईं। इसके अलावा, 58.3% जड़ रहित कट-शूटों में 0.5 मिलीग्राम/लीटर आईबीए प्रेरित रूटिंग के साथ ½ एमएस जोड़ा गया, जिन्हें अनुकूलन के लिए इन विट्रो और एक्स विट्रो तरीकों का पालन करके पौधों को पूरा करने के लिए कठोर किया गया था। एक एस एस आर मार्कर ईएमएफ21एम05 ने 20 पुनर्जनन कर्ताओं की युग्मक उत्पत्ति की पुष्टि की। बैंगन में आगे की फसल सुधार के लिए चौबीस पुनर्योजी पौधों की पराग मातृ कोशिकाओं को विभाजित करने में मेटाफ़ेज़ -1 चरण के साइटोलॉजिकल विश्लेषण ने पौधों को द्विगुणित (4), अगुणित (9), दोगुना-अगुणित (11) में वर्गीकृत किया।