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Micropropagation of *Mangifera indica* L. cv. Kurakkan through somatic embryogenesis

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Abstract

Nucellar embryogenesis was induced in *Mangifera indica* L. cv. Kurakkan, a polyembryonic salt tolerant, dwarfing rootstock. Nucellus tissue excised from 3.5 cm long fruits developed pro-embryonic callus in 19 days of inoculation on modified MS medium supplemented with 4.52µM 2,4-D, 0.05% malt extract and 13.78µM spermidine. Somatic embryogenesis exhibited high frequency (158.33 embryos). However, all the differentiated embryos proliferated on medium having low level of sucrose (4% w/v) and auxin (2.26µM 2,4-D). Most of the proembryonic calli converted into heart shaped and cotyledonary embryos by reducing temperature to 15°C. Somatic embryos were matured on modified MS medium fortified with 0.38µM ABA, 0.57µM IAA and 30.30µM PEG. Matured somatic embryos germinated (around 30%) on MS medium supplemented with 2.68µM NAA, 11.60µM kinetin and 2736.9µM glutamine.

Key words : Abiotic stress, Kurakkan, *Mangifera indica,* Polyembryonic, Rootstock, Salt tolerant, Somatic embryogenesis.

Introduction

Mango (*Mangifera indica* L.) is the most important fruit crop because of its wide adaptability, high nutritive value, richness in variety, delicious taste, excellent flavour, attractive appearance and commercial utility in India as well as in many part of the world. Kurakkan is a polyembryonic cultivar of mango, which is moderately resistant to salt, wind, and hoppers [1]. This could be an excellent rootstock for salt affected soils. Generally, most of the scion varieties are grafted on to heterozygous rootstock of seedling origin [2] resulting in non-uniform planting material. There is an urgent need to develop rapid and clonal mass multiplication system for this rootstock. Somatic embryogenesis is an efficient tool to augment mass scale propagation of various fruit crops. Further, a robust *in vitro* regeneration system in mango can facilitate genetic engineering.Somatic embryogenesis has been worked out in various mango cultivars *viz.*, Arka Anmol [3], Amrapali and Chusa [4, 5], Ambalavi [6] and Ataulfo [7] and Dashehari [8]. However, no work has been reported on Kurakkan so far. Therefore, present investigation has been undertaken to device a system for somatic embryogenesis in mango cv. Kurakkan.

Materials and methods

Young fruits of Kurakkan (2.5-4.5cm long) cultivars were collected 30-40 days after pollination from the germplasm block of Central Institute for Sub-tropical Horticulture, Rehmankhera, Lucknow. Fruits were washed under running tap water for 30 minutes. These were then dipped in solution containing 5.24µM Carbendazim (Bavistin) + 2-3 drops of Tween-20 for one hour and washed. The fruits were further sterilized with 3.68 µM $HgCl_2$ for 15 minutes followed by washing in sterile double distilled water for 5-6 times. The fruits were dipped in 70% alcohol for a few seconds and then flame sterilized at the time of inoculation under laminar airflow. There after the fruits opened and ovules were isolated under aseptic conditions and the ovules were bisected longitudinally. Intact ovular halves containing nucellar tissues were carefully scooped out and placed on induction medium (modified MS) [9]. The cultures were inoculated at 25±2°C, 50-55% RH. Initially the

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cultures were kept in dark. At the time of conversion, the embryos were shifted to 16 hrs light regime (40 mmol m^2s^{-1}quantum flux). Ten culture bottles formed one replication and each treatment was replicated thrice. The complete regeneration medium was divided into four categories (proliferation, conversion, maturation and germination medium). The details of medium used in the study are described below.

Embryo induction and proliferation medium

Modified MS medium contained full strength B-5 [10] major salts with full strength each of MS minor salts, MS iron-EDTA and MS organic supplements, 0.05% malt extract, 0.05% yeast extract, 2736.9µM L-glutamine, 4.52µM 2,4-D, cytokinin(s) (2.22µM each BAP, kinetin and zeatin separately), 567.76µM ascorbic acid, 2.5µM PVP (Hi-Media, India), 6% (w/v) sucrose (Qualigens Fine Chemical, India) and 0.8% (w/v) agar (Hi-Media, India). pH of the medium was adjusted to 5.8 prior autoclaving at 121^0C (15psi) for 15min. Ten culture bottles formed one replication and each treatment was replicated thrice. Frequently subculturing (at two days interval) was done to avoid oxidative browning of tissue. The number of explants showing callus and proembryonic calli initiation were recorded after hundred days. The data was statistically analysed using Complete Randomised Designing (CRD).

Conversion medium

After four weeks, proembryonic calli were transferred to conversion medium containing B-5 major salts, MS minor salts, MS iron-EDTA and MS organic supplements, 0.05% malt extract, 2736.9µM L-glutamine, 567.76µM ascorbic acid, 2.5µM PVP, 6% (w/v) sucrose and 0.8% (w/v) agar. The embryos were kept under different temperature regime (5, 15 and 25 ^{0}C) to asses conversion efficiency of embryos.

Maturation medium

Embryos of early or late heart shaped and early cotyledonary stage were formed on conversion medium These were transferred to maturation medium (Full strength B-5 major salts, full strength MS minor salts, iron-EDTA and organic supplements, abscisic acid (0, 0.19, 0.38 and 0.75µM) along with 0.57µM IAA, 30.30 µM PEG, 4% (w/v) sucrose and 0.8% (w/v) agar) after 4-6 weeks. Around 20 embryos were transferred to each culture bottle (50ml media). The cultures were incubated for 90 days at 25±2^{o}C, 50-55% RH and 16hrs light regime (40 mmol m^2s^{-1} quantum flux).

Germination medium

Mature embryos (>15.0 mm length) were transferred individually to 20 ml medium in test tubes containing half strength B-5 major salts, MS minor salts, iron-EDTA and organic supplements, 2.68µM NAA, 11.60µM Kinetin, 2736.9µM L-glutamine, 2.88µM GA_3, 4% (w/v) sucrose and 0.8% (w/v) agar. The cultures were exposed to 25±2^0 C temperature for 16 hrs photoperiod with light at 40-m mol m^2 s^{-1} quantum flux at 55% relative humidity. Germinated somatic embryos (which shows shoot and root primordia) were recorded after one month.

Root promotion media

The functional roots from somatic embryos were obtained by transferring the germinated embryos in the rooting medium (full strength B-5 major salts, full strength MS minor salts, iron-EDTA and organic supplements, 9.80µM IBA, 2.68µM NAA, 2.5 µM PVP, 13.78µM spermidine, 3% (w/v) sucrose and 0.8% (w/v) agar) followed by partial root cutting.

Results and discussion

Size of explant

The type and size of explant has been reported to be correlated with the *in vitro* responses in mango. It is clear from the data (Table 1) that nucellar tissues excised from 3.5 cm long fruits (Fig. 1) triggered higher production of proembryonic calli (44%) leading to formation of large number of somatic embryos (158.33). Nucellus excised from longer and smaller fruits did not produce good proembryonic calli. Immature fruits around 2-3 cm long were ideal for culture initiation in 'Arka Anmol' [3]. The efficiency of regeneration from nucellar tissue of mango has been genotype dependent in polyembryonic cultivars [11, 12]. The size of explant to be cultured is of great importance. The larger the explant, poor in the response. In this context the medium has a limited influence. On the other hand, small explants are more easily directed by the substances contained in the medium [13].

Embryo induction and proliferation

Analysis of the obtained data (Table 2) revealed that major salts of B-5 and minor salts of MS containing 4.52µM 2, 4-D, 13.78µM spermidine, 0.05% malt-extract, 2736.9µM glutamine and 0.05% yeast extract augmented somatic embryogenesis in mango. As many as 158.33 embryos per culture could be produced using this treatment. Proembryonic calli could be developed

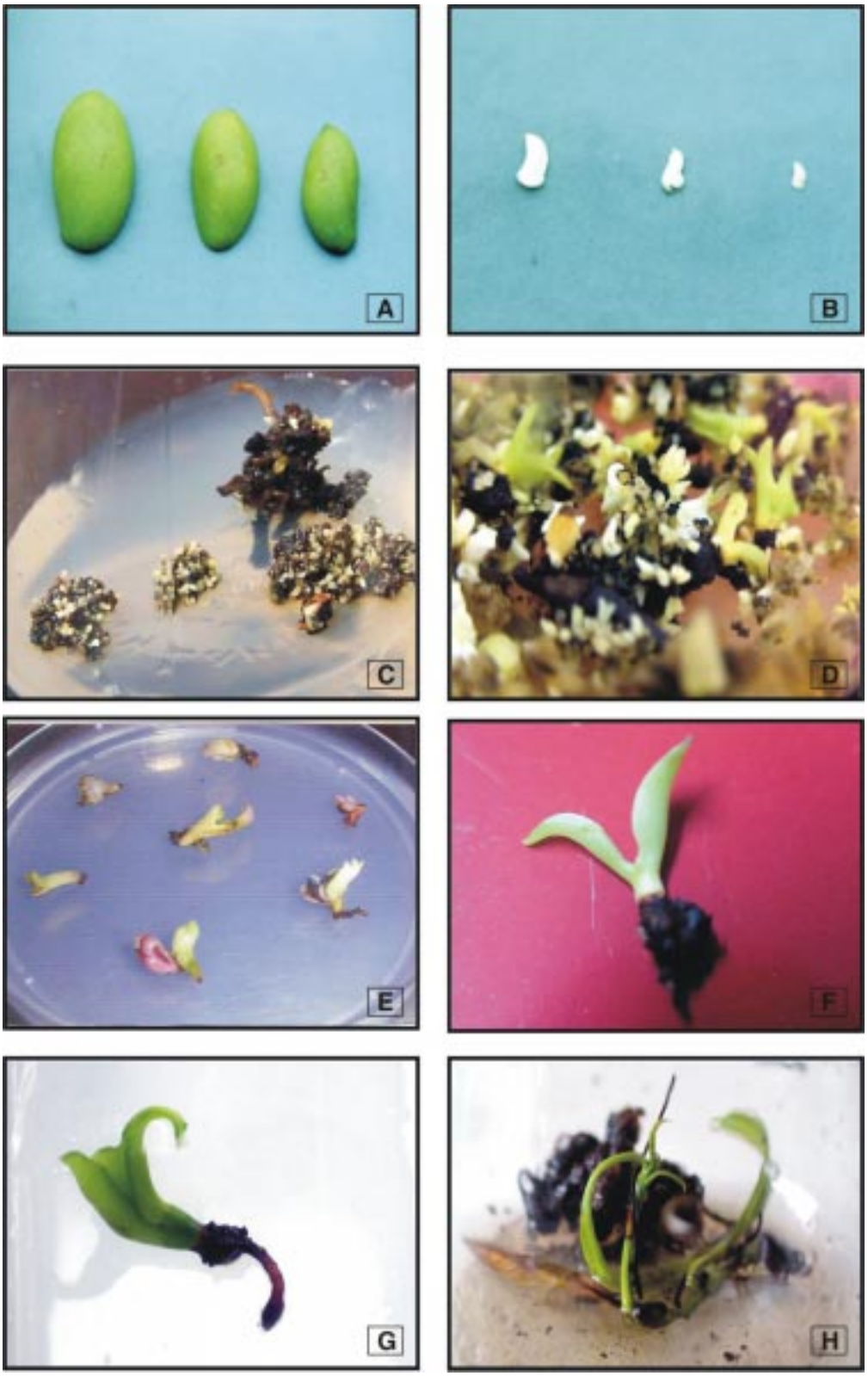

Fig. 1. Different stages of somatic embryogenesis in mango (*Mangifera indica* L) var. Kurukkan. A-Different size of fruits (2.5 -4.5cm long), B- excision of nucellar embryos, C-development of globular embryos, D-conversion of SE into heart shaped, E- early cotyledonary stage, F-late cotyledonary shaped embryo, G-rooting and conversion into early stage of plants and H-rooted plants growing vigorously in liquid culture medium

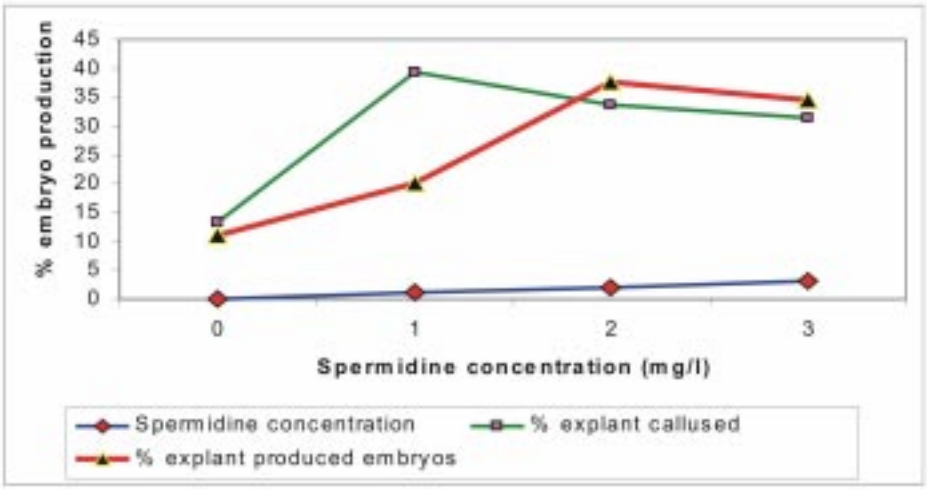

Fig. 2. Influence of Spermidine on production of somatic embryo in Mango cv. Kurrukan

Table 1. Effect of size of explant on somatic embryogenesis in Kurakkan

Size of explant(cm)	Days taken for callusing	% explant with pro-embryonic calli	Days taken for somatic embryo initiation	Number of embryos after 100 days
2.5	22.5	40.44	49.5	31.66
3.5	18.3	44.44	47.5	158.33
4.5	19.5	33.33	46.5	64.66
SEm ±	0.89	1.75	0.84	7.43
CD (P=0.05)	1.73	3.40	1.63	14.44

Table 2. Mean Effect of bioregulants and media additives on somatic embryogenesis in Kurakkan

Treatments (μM)	Days taken for initiation of PEC	Days taken for embryos initiation	No. of embryos	Fresh weight of embryos (gm)	Dry weight of embryos (gm)
Basal	0.0	0.0	0.0	0.0	0.0
Basal + 4.52 2,4-D + 13.78 spermidine	20.6	45.3	119.5	0.1346	0.0621
Basal+ 4.52 2,4-D + 13.78 spermidine +2.22 BAP	23.6	46.3	84.55	0.1278	0.0671
Basal + 4.52 2,4-D + 13.78 spermidine +2.32 Kinetin	25.6	503	75.33	0.1008	0.0535
Basal + 4.52 2,4-D + 13.78 spermidine + 2.28 Zeatin	27.0	50.6	62.14	0.6647	0.0886
Basal +4.52 2,4-D + 13.78 spermidine + 0.05% yeast extract	24.6	53.0	23.33	0.0922	0.0396
Basal + 1.0 2,4-D + 13.78 spermidine + 0.05% Malt extract	19.0	33.3	158.33	0.1752	0.0918
SEm±	0.682	1.056	4.36	0.075	0.006
CD (P=0.05)	1.20	1.86	7.68	0.132	0.011

in just 19 days under this treatment, followed by media containing 4.52µM 2, 4-D and 13.78µM spermidine (20.6 days). This treatment also reduced the time required for initiation of somatic embryos (33.3 days), followed by the media containing 4.52µM 2, 4-D, 2.22µM BAP and 13.78 µM spermidine (46.3 days). It seems that incorporation of spermidine and malt extract is crucial for augmentation of proliferation of somatic embryos. Exogenous supply of spermidine increased embryogenic potential of nucellar tissue when used alone or in combination with other polyamines [14]. Different levels of spermidine (0-20.67µM) used along with 4.52µM 2, 4-D and 0.05% malt extract (Fig.2) revealed that 13.78µM spermidine is prerequisite for quick initiation and proliferation of somatic embryos. Litz and Yurgalevitch [15] reported that regulation of ethylene biosynthesis and spermidine synthase in mango cultivars may be the most significant factors that controls somatic embryogenesis pathway.

Embryo conversion

It is evident from observed data that temperature stress is helpful in conversion of somatic embryos (Table.3). The early heart shaped embryos when inoculated in conversion medium and kept under different temperature regime revealed that at 15^{o}C the embryos shows good conversion efficiency (28.66) as compared to control (25^{o}C). At lower temperature most of early heart embryos converted into late heart and cotyledonary embryos. However, longer exposure of embryos in low temperature delays the germination efficiency of embryos. Lower incubation temperatures (5 and 15^{o}C) delays germination process [16] in mango.

Maturation and germination

Well developed (>15mm) cotyledonary embryos matured on full strength B-5 major salts, full strength MS minor salts, iron-EDTA and organic supplements, 0.38µM abscisic acid along with 0.57µM IAA. Supplement of abscisic acid is pre-requisite for maturation of embryos. Matured embryos were cultured on medium containing half B-5 major salts, full strength MS minor salts, iron-EDTA and organic supplements, 2.68µM NAA, 11.60µM Kinetin, 2736.9µM L-glutamine, 2.88µM GA_3, 4% (w/v) sucrose and 0.8% (w/v) agar. Around 30% embryos germinated within 15 days. Single black root emerged from root primordia followed by development of leafy shoot. This *in vitro* developed root had no root hair, vascular connection was poorly developed. All the rooted plants were shifted to autoclaved coconut husk containing MS Salt mixture and kept at acclimatization chamber at 30^{o}C and 75% RH. Almost all the plants developed apical necrosis within a month after shifting and died. Somatic embryogenesis protocol can be gainfully utilized for development of transgenic mango. Transformed embryos can be converted easily in to healthy shootlets which can be micrografted onto a suitable rootstock.

Table 3. Effect of temperature on conversion of early heart shaped embryo into late heart shaped embryo after 30 days

Temp. ^{o}C	% embryos converted to heart shaped	% embryos necrosed	% embryos survived
5	0.00	36.33	63.67
15	40.94	8.66	91.34
25	34.76	10.66	89.34
SEm±	0.75	1.02	1.32
CD (P=0.05)	1.46	1.98	2.57

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