Pak. J. Bot., 33(2): 211-215, 2001.

NUCELLAR REGENERATION AND POLYEMBRYONY OF CITRUS CULTIVARS

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Abstract

Thirty four *Citrus* cultivars were studied for nucellar embryogenesis and for the extent of polyembryony per seed. Ovule weight had no correlation with the number of nucellar embryos. Also seed weight had no correlation with the number of embryos per seed. The maximum and minimum number of embryos per nucellus were in cvs. Kinnow (7.75) and Baramashi (2.23), respectively. Secondary somatic embryogenesis was also studied which was maximum in cv. Kinnow (8.40) and minimum in cv. Baramashi (2.90). Maximum and minimum polyembryony were in cultivars Minneola (7.50) and Orlando (2.75), respectively which indicated that nucellar embryogenesis was cultivar dependent. The soil survival after two months hardening process was maximum in cvs. Minneola (100 %), Shamber (100 %), Foster (100 %), Gada dehi (100 %) and minimum in cv. Baramashi (10.00%).

Introduction

Citrus is a prized fruit of Pakistan and holds number one position among all fruits both in area and production. At present about 196,000 hectares are under *Citrus* fruits with a total production of 2,037,000 tonnes which is about 31% and 32% of total fruit area and production of the country (Anon.,1998). Of a total of 95% of country's total *Citrus* fruits production, Punjab produces Kinnow (61.65), mandarins (5.11), Mosambi (10.09), Sweet oranges (13.11), Sweet lime (3.88), Lemon (1.65), Sour/Kaghzi lime (1.45), Sour orange (0.95), Grape fruit (0.10) and other *Citrus* fruits (2.01) (Anon., 1999).Pakistan earns about Rs. 650 million every year by exporting Kinnow fruits to South-East Asian and Far-Eastern countries (Anon., 2000).

Nucellar somatic embryogenesis is well recognized in *Citrus* species (Starrantino *et al.*, 1993). It is preferred for (i) clone propagation because it is the least differentiated mother tissue, (ii) since nucellus is at single cell stage till 100-120 days after pollination (DAP), it can be mutagenized before embryogenesis at single cell stage for access of solid mutants which avoid complications of chimera and further bud wood selection, (iii) culture of immature ovules allows the production of nucellar plantlets from seedless cultivars, (iv) nucellus is virus free and so the nucellar embryos, (v) nucellar embryos are self rooted which avoid complications of graft incompatibilities.

The quality of *Citrus* fruit is influenced by both rootstock and management practices (Nieves *et al.*, 1991). Scions prepared through nucellus culture are usually grafted onto soil adapted rootstocks because of (i) early fruiting, (ii) avoidance of juvenility problems, (iii) uniform tree size, (iv) control of production and fruit quality, (v) tolerance to stress factors like salinity, high pH, viral, bacterial, fungal and parasitic nematodes. The objective of the present work was to ascertain the nucellar embryogenic capacity of specific cultivars and to find if ovule weight has any relation with the number of nucellar embryos produced, seed weight with polyembryony counts or if the number of embryos are genotype dependent.

Materials and Methods

The experiment was conducted at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad during 1998-2000. The experimental materials as given in Table-1 were collected from different *Citrus* orchards of Faisalabad region.

Nucellus culture: Immature fruits of 45-90 days were collected. The fruits were washed thoroughly and surface sterilized by immersing in 70% ethyl alcohol for few seconds and flamed. These fruits were dissected in a laminar air flow bench and normal ovules were extracted. Both the ovule integument's were removed and nucellus tissues were cultured in flasks containing MS medium (Murashige & Skoog, 1962) supplemented with 0.5 mg/l BA and 5 mg/l glutamine. The medium was solidified with 1% Difco agar and the pH was adjusted at 5.5-5.8, prior to autoclaving. Forty ml of culture medium was dispensed in 100 ml flasks and sterilized by autoclaving. Five nucellus tissues were cultured in one flask. The cultures were kept in a room with 1000 lux light\ 8 hours dark cycle at temperature of 25 ± 2°C. The data for regeneration of primary embryos per nucellus was recorded after two months of culture period, while for secondary embryogenesis, the data was recorded after three successive subculturing.

Shoot apices of *in vitro* raised nucellar embryos of *Citrus* cultivars Ruby red, Frost navel, Blood red, Chinese lemon, Eureka lemon and Grape fruit were grafted onto rootstock seedlings of rough lemon, Kherna khatta and Gada dehi. Humidity was maintained by polythene bags. The results were recorded after one month of grafting. Humidity was gradually released after 2 months of grafting with emergence of new leaves. Five grafts of each cultivar were made.

Polyembryony: Fully ripened fruits were utilized during this study. After washing with tap water all the fruits were cut into halves and extracted fully developed seeds. These seeds were washed with tap water to remove the attached gelatinous materials and surface dried. The upper testa of seeds was removed and then were sterilized with 0.01 % $HgCl_2$ for one minute. These seeds were rinsed 3-4 times with autoclaved tap water and soaked in flasks containing distilled water for 30 minutes to soften their inner testas. The inner testa were scratched, to facilitate embryo germination. These seeds were cultured in test tubes containing 10 ml MS medium supplemented with 1 mg/l GA_3 and 5 mg/l glutamine. This medium was solidified with 1% Difco agar and the pH was adjusted at 5.5-5.8 before autoclaving. These cultures were kept at room temperature. After 35 days, germinated embryos per seed were counted and then transferred to soil mixture in pots. After two months of hardening process, the survival percentage of embryos in soil was recorded.

Results

Nucellus embryogenesis and seed polyembryony of 34 *Citrus* cultivars including sweet oranges, mandarins, hybrid cultivars, lime, lemons, grape fruit, pumelo and rootstocks are given in Table 1. Cultivars Sanguinello (0.90 g), Mediterranean (0.60 g), Shamber (0.53 g) and Chakotra (0.52 g) have more ovule weight, more nucellus mass and so the larger initial explant tissue to start with but the number of primary embryos counted in these cultivars were 3.80, 4.50, 3.50, 3.60 respectively.

Table 1. Nucellus tissue derived embryos and polyembryony of *Citrus* cultivars.

Cultivar	Ovule Wt. (g)	Embryos/Nucellus *P.E.	Embryos/Nucellus *S.E.	Seed Wt.(g)	Polyembrony/ Seed	Survival %
Mosambi	0.11	5.50	5.60	0.139	6.25	45.45
Frost navel	0.28	4.50	5.50	0.910	4.83	81.25
Washington navel	0.19	3.50	5.70	0.150	4.17	53.85
Ruby red	0.22	3.50	5.70	0.162	6.83	83.33
Succari	0.20	4.00	5.90	0.17	3.83	76.92
Hamlin	0.45	4.10	5.00	0.176	5.00	69.23
Jaffa	0.40	6.50	8.00	0.144	7.09	63.64
Valencia	0.39	5.80	7.00	0.154	5.42	69.23
Moro blood	0.15	2.50	4.15	0.225	2.83	70.00
Tarocco	0.32	4.80	6.17	0.229	4.17	50.00
Mediterranean	0.60	4.50	3.68	0.198	2.83	40.00
Blood red	0.48	5.50	6.50	0.157	5.75	50.00
Sanguinello	0.90	3.80	5.00	0.155	3.25	66.67
Pineapple	0.44	4.50	6.00	0.156	6.75	66.67
Orlando	0.15	4.83	5.70	0.123	2.75	70.00
Kinnow	0.13	7.75	8.40	0.152	5.24	69.23
Fewtrel's early	0.06	6.17	6.35	0.151	4.42	54.55
Honey	0.18	4.17	4.60	0.161	3.00	33.33
Pixie	0.04	4.50	4.70	0.122	4.50	69.23
Ponkan	0.07	3.17	3.70	0.132	3.16	76.92
Tangerine	0.31	3.50	4.19	0.151	5.17	83.33
Minneola	0.29	5.17	5.50	0.130	7.50	100.00
Seminole	0.08	4.50	5.30	0.156	4.83	92.86
Sweet lime	0.09	5.17	5.88	0.160	5.25	62.50
Chinese lemon	0.05	3.50	4.50	0.101	3.50	50.00
Baramashi	0.06	2.23	2.90	0.110	3.00	10.00
Eureka lemon	0.14	3.17	4.00	0.089	3.50	84.62
Shamber	0.53	3.50	5.60	0.162	2.90	100.00
Duncan	0.45	4.90	4.30	0.150	2.90	80.00
Foster	0.10	5.17	5.50	0.175	2.83	100.00
Chakotra	0.52	3.17	3.60	0.261	2.83	73.33
Rough lemon	0.10	5.17	5.98	0.139	5.20	73.33
Gada dehi	0.09	5.50	6.35	0.181	7.00	100.00
Kharna Khatta	0.14	3.50	4.83	0.137	6.16	80.00

*P.E. = Primary embryo, S.E. = Secondary embryo.

Kinnow (7.75), Jaffa (6.50), Fewtrell's early (6.17), Valencia (5.80), Mosambi, Blood red and Gada dehi (5.50) had good aptitude of forming embryos in primary culture. Cultivars Baramashi (2.23), Moro blood (2.50), Ponkan, Eureka lemon, Chakotra (3.17), Kharna khatta, Shamber, Chinese lemon, Washington navel, Ruby red and Tangerine (3.50) generated fewer embryos in primary nucellus culture.

The secondary nucellar embryogenesis response was good in Kinnow (8.40), Jaffa (8.00), Blood red (6.50), Fewtrell's early (6.35), Gada dehi (6.35), Tarocco (6.17), Pineapple (6.00), Rough lemon (5.98) and Succari (5.90), while Baramashi (2.90), Chakotra (3.60), Mediterranean (3.68), Ponkan (3.70), Eureka lemon (4.30) and Tangerine (4.19) were less responsive.

Immature ovule weight, primary and secondary nucellar embryos had no correlation with each other and hence with the seed weight and polyembryony. The number of embryos in specific culture environment are genotype dependent. In micro-grafting, the embryonic shoot apices wilted within one week if there was no union between shoot apex and root stock seedling tissues. Lock and key type fitting and union of cone shaped basal cut of shoot apex and cutting of V-shaped notch at the top of seedling is an art and experience to some extent. However, healthy scion embryos with normal shoot, root and cotyledon development are more easily grafted and showed signs of growth and development after 2 weeks. The best response of micro-graft was of Ruby red on Sour orange cultivar Gada dehi followed by Chinese lemon on rough lemon, Mosambi and Frost navel on Sour orange Cv. Kherna khatta, Eureka lemon and Grape fruit on rough lemon seedlings. The plants require hardening procedure after 2 month graft period with the emergence of new leaves otherwise they wilt with the sudden decrease of humidity.

Cultivars with larger seed size and weight were Frost navel (0.910 g), Chakotra (0.261 g), Tarocco (0.229 g) and Moro blood (0.225 g) with the 4.83, 2.83, 4.17, 2.83 embryos per seed respectively in specific medium and time period. Maximum polyembryony was observed in Minneola (7.50), Jaffa (7.09), Gada dehi (7.00), Ruby red (6.83) and Pineapple (6.75).

Plants arising from a single polyembryonic seed ranged in size from very small to typically large. Large seedlings from different seeds also varied in size.The normal embryos with balanced shoot root system survived in soil and the seedlings of Gada dehi, Shamber, Foster, Minneola, Valencia, Rough lemon cv.Jatti khatti and Kinnow were more adaptable and healthy with 100, 100, 100, 69.23, 73.33 and 69.23 % survival, respectively.

Discussion

Nucellar embryogenesis have been exploited by various workers (Takashi *et al.*, 2000) using pre-anthesis and post anthesis ovules in *in vitro* and *in vivo* culture (Perez *et al.*, 1998).Most of the commercially important *Citrus* species are polyembryonic because of *in vitro* nucellar embryogenesis. A seed may contain several nucellar embryos of maternal genotype and a possibility of single zygotic embryo. Nucellar embryony is a hinderance to *Citrus* breeding as in some crosses no sexual seedling survive (Moore & Castle, 1988). Few or no zygotic seedlings are produced when polyembryonic cultivars are used as maternal parent, because nucellar embryos restrain and often abolish zygotic embryo development prior to seed maturation (Kobayashi *et al.*, 1988). Three types of polyembryony have been suggested so far: (1) polyembryony of nucellar cells which develop within the female gametophyte giving rise to adventitious embryos of true to type (2) monozygotic polyembryony by cleavage of the sexual embryo resulting in phenotypically identical twin hybrids from single seeds and (3) two gametophytes in a single ovule leading to dizygotic, non-identical twins, this phenomenon although infrequent has implications for the evolution of genus and for *Citrus* breeding (Medina Filho *et al.*, 1993).

Citrus isozyme methods are used to identify various genotypes (Protopapadakis & Papanikolaoss, 1998). Interspecific hybrid combinations are easily distinguishable from isozyme genotypes from their nucellars but hybrids between varieties of the same *Citrus* species can not be well separated as they have the same isozyme genotypes and so the zygotics and nucellars within the cultivar are not sharply detectable by isozyme genotypes.

Cultivar genotype plays key role in the production of nucellar embryos but the weight of ovule or size of nucellus tissue excised or seed weight has no influence on polyembryony. However, it was observed in this study that healthy seeds, vigorous growth of plants with heavy bearing had positive influence on polyembryony for a specific cultivar.

Seed size has clear effects on seedling vigour, it did not seem to be related to the genotype of the embryo. Nucellar seedlings were generally more vigorous than zygotics (Xiang & Roose, 1988). Bowman *et al.*, 1995 found that seed size and shape were related to the number of seedlings produced and the likelihood of recovering a zygotic seedling. The relationship between seed size and shape and likelihood of recovering a zygotic seedling most often was connected with the weight or thickness of a seed in their studies. Primary and secondary somatic embryos, polyembryony, embryo soil survival and micro-grafting success, all are cultivar dependent. Micro-grafting of shoot apical meristem onto well soil adapted rootstock seedlings is the best procedure for soil transfer and over-coming the problem of nursery transplant of *in vitro* regenerated material (Hazarika *et. al.*, 1999). The acclimatization of micropropagated plantlets can be made efficiently by various nutrient additions to soil (Parthasara *et. al.*, 1999).

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(Received for publication 10 March 2001)