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Polymorphism influences seed viability and vigour in poly-embryonic Kaffir lime (*Citrus hystrix*) seeds

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Abstract

Polyembryonic seeds of Kaffir lime showed polymorphism for number of embryos, their viability and the vigour of the seedlings. This polymorphism was determined by the embryo size, weight and accumulation of reserve materials. In general, the seed has a maximum of 5.4 embryos with an average of 2.9. Correlation was observed between the seed size and the number of embryos: heavier seeds possessed more embryos. Tetrazolium viability staining was positively correlated with the number of seedlings produced from the polyembryonic seeds. The viability of the embryos varied within a seed with some embryos viable and the rest non-viable reflecting the number of seedlings produced per seed that was less than the number of embryos actually present. Significant differences were also noticed between single seedlings that possessed higher vigour than seedlings arising from polyembryonic seeds.

Introduction

Polyembryony is an important phenomenon in *Citrus* species in which two or more embryos are present in a single seed. Such embryos may be of sexual origin (gametophytic polyembryony) or of somatic or nucellar origin (sporophytic polyembryony). Gametophytic polyembryony includes apogamety and apospory, whereas nucellar, integumental, monozygotic cleavage and endospermal polyembryony are grouped under sporophytic polyembryony (Batygina and Vinogradova, 2007). Sexual polyembryony is due to multiple fission of the zygotic embryo or the presence of more than one egg in an ovule (Bacchi, 1943). Some species have been reported to produce two or more sexual embryos in one seed (Ozsan and Cameron, 1963).

During embryo sac expansion, nucellar embryos develop alongside the zygotic embryo, which may or may not develop fully. Thus the number of embryos produced from a seed may vary among cultivars (Altaf *et al*., 2001). Cultivar genotype plays a key role in the production of nucellar embryos but the weight of ovule or size of nucellus tissue has no influence on polyembryony (Altaf *et al*., 2001).

Kaffir Lime (*Citrus hystrix* DC.) is an evergreen tropical tree grown for its fruits and leaves which are used to flavour Thai dishes, soups and curries. The acidic fruit juice is added to fish and poultry dishes in Malaysia and Indonesia. Fruits are pickled in India and are rich in antioxidants. Kaffir lime is mainly propagated from seed but also by vegetative means. In the case of seed propagation, the seeds are sown fresh to get higher germination. The species has polyembryonic seeds in which the number of seedlings produced varies between seeds. Similarly, seedling vigour differences can be observed during germination and seedling establishment. This may be due to the differences in the vigour and viability of the polyembryos. However, research on the influence of polymorphism on seed viability and vigour of this polyembryonic species is lacking. Therefore, attempts were made to determine the morphological and physiological variations in the embryos and their influence on regeneration ability.

Materials and methods

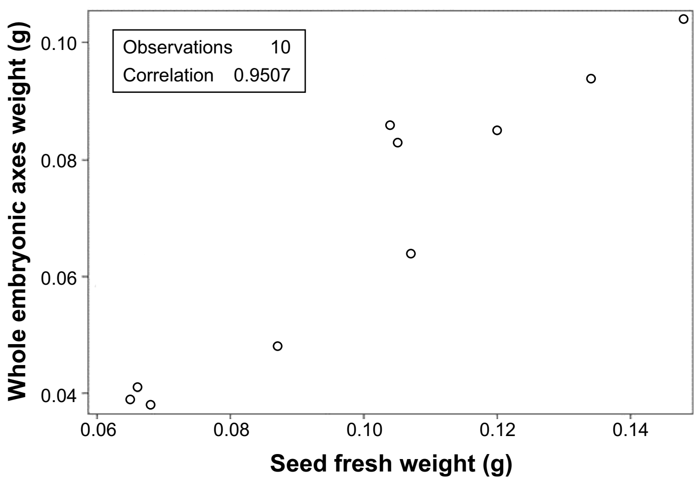
Fully ripe yellow fruits were collected from Kaffir lime trees located at Rasipuram (India) and the seeds were extracted by cutting and squeezing the fruits. Ten seeds were selected randomly from the bulk. The selected seeds were surface-dried and weighed before and after decoating, and examined under a microscope to separate each embryo along with the cotyledons. The number of embryos present in each seed was counted and weighed.

The viability of embryos was assessed by taking 50 randomly selected seeds from the bulk. The seeds were placed in 10 beakers each comprising five decoated seeds. The seeds were soaked in 0.5% 2,3,5-triphenyl tetrazolium chloride (TTZ) solution prepared using phosphate buffer. The seeds in the solution were placed in an incubator maintained at 40°C for staining for one hour. Then, stained seeds were removed from the solution and washed thoroughly for observation of the staining pattern and viability (Moore, 1972). Similarly, the excised embryo test (EET) was conducted using 50 randomly selected excised embryos obtained from fresh seeds. The embryos were placed in 10 Petri dishes comprising five in each plate with paper medium. The number of emerged embryos in each plate was counted 15 days after the test to determine percentage viability.

Polyembryonic seedlings was observed by placing 10 seeds in sand and placing in a germination cabinet maintained at 25°C and 90% relative humidity. At the end of the germination test (30 days), the number of seeds germinated was counted. Mean germination time (MGT) of the mono- and polyembryonic seeds was calculated by the formula $MGT = \Sigma\ (ni/di)$ in which, ni is the number of germinated seeds and di is the day of counting (Ellis and Roberts, 1981). The number of radicles emerged in each seed was counted 15 days after commencing the germination test. The number of survived seedlings was counted 30 days after the germination test. Then, the seeds were grouped into five categories based on the number of seedlings produced by each seed. Seedling length and fresh weight were measured. Correlation analysis was performed using Statistical Analysis System (SAS) software (SAS Institute Inc.).

Results

The fresh weight of individual seeds ranged from 0.065 to 0.148 g and embryo weight from 0.104 to 0.038 g (figure 1). Larger seeds (0.148 g) had the most embryos (5) and the smallest seeds (0.065 g) had just one embryo. There was an overall average of 2.9 embryos in a seed (figure 2A, B). The embryonic axes of embryos from seeds with the most embryos were smaller (0.013 to 0.036 g) while the weight of axes of embryos from seeds with e.g. four embryos was larger (0.005 to 0.035 g) (table 1). Seeds with two embryos, had one larger embryo (0.034 to 0.084 g) and one smaller embryo (0.001 to 0.034 g).



**($P < 0.01$)

Figure 1. Seed fresh weight and weight of embryonic axes in *Citrus hystrix*.

Table 1. Differences in polyembryonic axis weight in *Citrus hystrix*.

Seed No.	Seed fresh wt. (g)	No. of polyembryonic axes	Polyembryonic axis fresh wt. (g)				
			Embryo 1	Embryo 2	Embryo 3	Embryo 4	Embryo 5
1	0.107	4	0.035	0.015	0.009	0.005	–
2	0.134	2	0.060	0.034	–	–	–
3	0.120	2	0.084	0.001	–	–	–
4	0.105	2	0.060	0.023	–	–	–
5	0.148	5	0.036	0.023	0.018	0.014	0.013
6	0.065	1	0.039	–	–	–	–
7	0.087	3	0.027	0.019	0.002	–	–
8	0.104	2	0.077	0.009	–	–	–
9	0.066	2	0.040	0.001	–	–	–
10	0.068	2	0.034	0.004	–	–	–

The size of the cotyledonary leaves also differed. Among two cotyledonary leaves, one was larger than the other (figure 2C). When the embryos were stained by 2,3,5-triphenyl tetrazolium chloride solution, seeds with a single embryo stained well (figure 2D). For seeds with two or more embryos, some embryos were either unstained or partially stained (figure 2E). In the case of seeds with three embryos, there was generally one fully-stained embryo and the remaining had unstained or partially stained axes (figure 2F). It was also noticed that the larger embryos were more likely to be fully stained than the smaller ones (figure 2F). Nevertheless, 100% germination was observed in all the seeds. The viability by 2,3,5-triphenyl tetrazolium chloride staining was 80%. Similarly, maximum viability of 64% was obtained by the excised embryo test (figure 3).

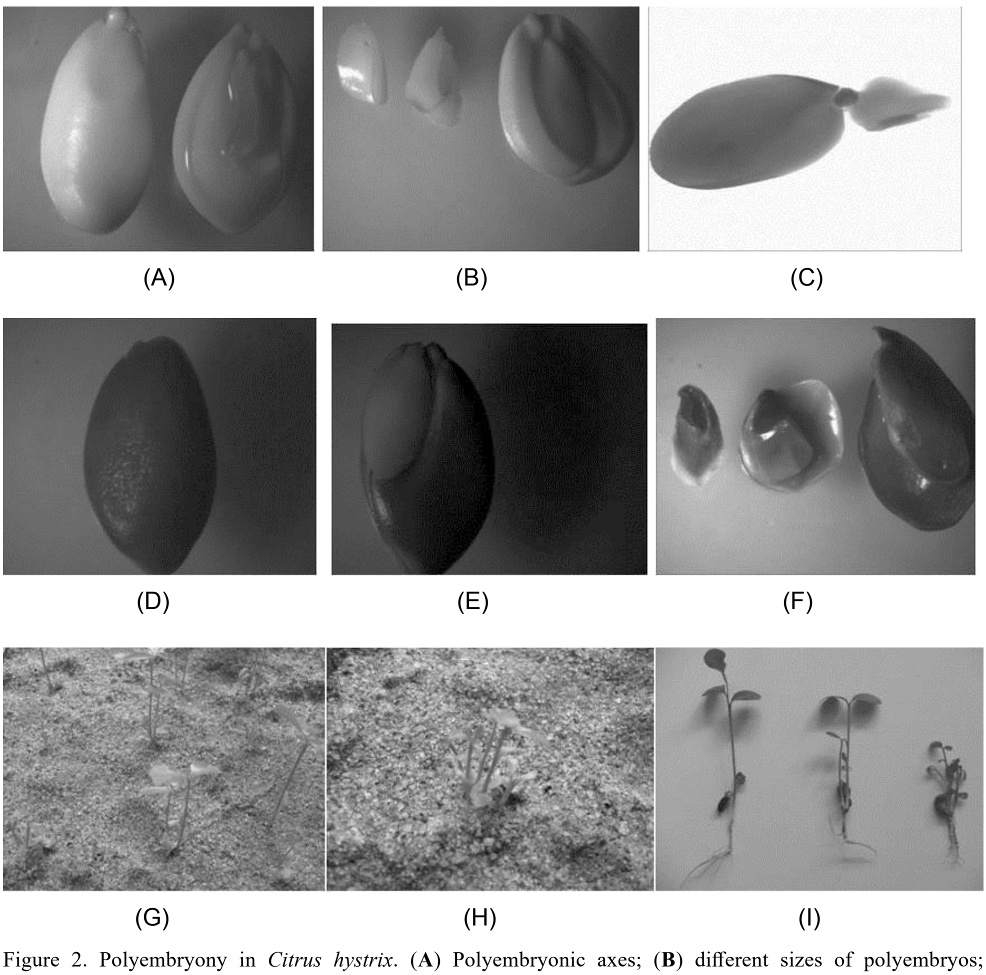


Figure 2. Polyembryony in *Citrus hystrix*. (**A**) Polyembryonic axes; (**B**) different sizes of polyembryos; (**C**) embryonic axes with different cotyledonary size; (**D**) fully stained embryo; (**E**) stained and unstained embryos; (**F**) fully stained and partially stained embryos; (**G**) polyembryonic seedlings; (**H**) five seedlings from a single seed; (**I**) differences in vigour of polyembryonic seedlings.

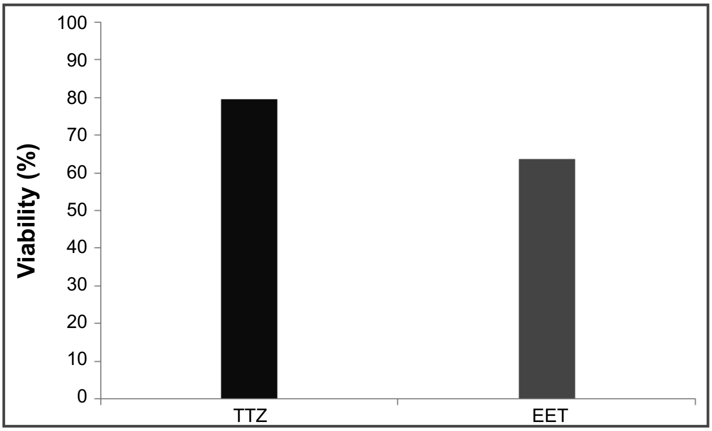


Figure 3. Viability of the embryos by tetrazolium staining (TTZ) and excised embryo test (EET) in polyembryonic seeds of *Citrus hystrix*.

There was a significant correlation between the number of tetrazolium-stained embryos and the number of emerged seedlings in the germination test (figure 4). The number of seedlings produced was 1 or 2 with a mean of 1.24. Mean germination time (MGT) of the seedlings emerged in mono- and polyembryonic seeds showed that the seeds with one embryo were faster to emerge (8.0 days). The MGT of the seedlings increased as the number of embryos increased. The second and subsequent seedlings took more time to emerge (figure 2G). The fifth seedling of the polyembryonic axis recorded the longest time to protrude (27.5 days; table 2). Similarly, the seeds that produced only one seedling had higher seedling length (150 mm) and fresh weight (0.260 g) when compared with seeds

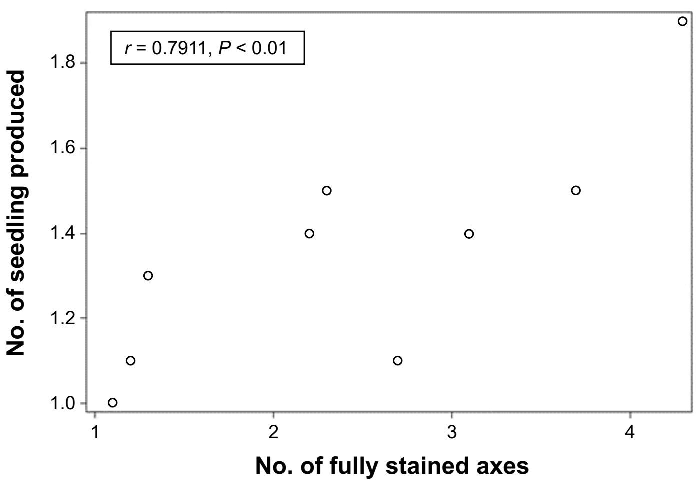


Figure 4. Tetrazolium staining and seedling production in *Citrus hystrix*.

Table 2. Mean germination time of polyembryonic axes in *Citrus hystrix*.

No. of axes seedling^{-1}	Mean germination time (days)				
	Seedling 1	Seedling 2	Seedling 3	Seedling 4	Seedling 5
1	8.0	–	–	–	–
2	8.7	14.0	–	–	–
3	8.5	13.0	20.0	–	–
4	8.5	15.5	19.0	23.5	–
5	10.5	13.5	19.0	23.5	27.5

with more than one seedling (table 3). Seedling vigour was poor when five seedlings were produced from a single seed (figure 2H, I). Polyembryos gave rise to shorter and lighter seedlings (table 3).

In general, all the seedlings emerged from the polyembryos survived when the number of seedlings was one or two. If the seedling number was ≥ 3, the survival rate reduced (figure 5). Nevertheless, an average of 2.1 survived seedlings was recorded from the 2.6 seedlings that emerged.

Table 3. Seedling vigour of polyembryonic seeds in *Citrus hystrix*.

No. of axes seedling^{-1}	Seedling length (mm)				
	Seedling 1	Seedling 2	Seedling 3	Seedling 4	Seedling 5
1	150	–	–	–	–
2	101	93	–	–	–
3	129	103	20	–	–
4	82	74	43	41	–
5	71	58	57	46	40
No. of axes seedling^{-1}	Seedling fresh wt. (g)				
	Seedling 1	Seedling 2	Seedling 3	Seedling 4	Seedling 5
1	0.260	–	–	–	–
2	0.194	0.087	–	–	–
3	0.162	0.135	0.018	–	–
4	0.103	0.077	0.059	0.021	–
5	0.049	0.041	0.036	0.037	0.036

Discussion

The presence of polyembryo in *C. hystrix* is dependent on seed weight and size. Such dependency was also recorded by several workers (Ozsan and Cameron, 1963; Cameron and Garber, 1968; Koltunow *et al*., 1995; Altaf *et al*., 2001; Aleza *et al*., 2010) in other

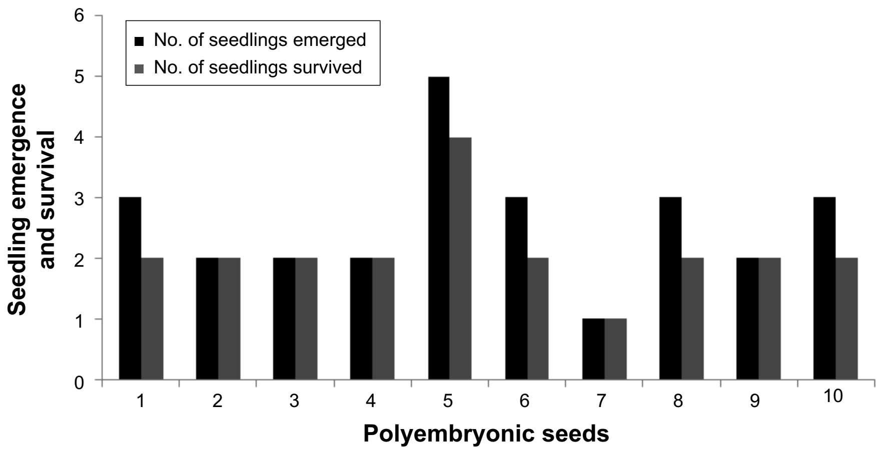


Figure 5. Emergence and survival of seedlings in polyembryonic seeds of *Citrus hystrix*.

citrus species. Soares Filho *et al*. (1995) observed 1 to 12 embryos per seed in citrus, with an average of 2.7 embryos. According to Altaf *et al*. (2001), the ovule weight and seed weight had no correlation with the number of nucellar embryos. They also opined that the production of polyembryos are cultivar specific, however, healthy seed, vigorous plant growth and heavy bearing had positive influence on polyembryony. Similarly in *C. hystrix*, the more embryo weight could be attributed to the metabolic potential of larger seeds that were able to provide nutrition to all the embryos leading to lower weight range in larger seeds and vice versa. In such cases, the larger embryos might be nucellar embryos and the smaller true embryos. In any case, for seeds with a single embryo, it must be a true embryo derived from fusion of gametes.

Polyembryony is also affected by the type of pollinator (Soares Filho *et al*., 1995). Any factor that affects pollination, fertilisation or seed development will also affect the percentage of polyembryony and embryo number per seed. However, Bowman *et al*. (1995) found that the size and shape were related to the number of seedlings produced. In species producing monoembryonic seeds, usually only one embryo remains in a mature seed as the result of competitive development; any others die during the early developmental stages (Batygina, 2005). The number of embryos per seed was negatively correlated with embryo length, implying that as the number of embryos per seed increased, embryo size decreased. This may be due to competition for nutrients among embryos (Andrade *et al*., 2004). Competition between embryos for nutrients could have resulted in slower development of zygotic embryos with nucellar embryos outgrowing their zygotic sibling as they form earlier (Wakana and Uemoto, 1988; Koltunow, 1993). Sometimes, competition for nutrition leads to differences in the size of the cotyledonary leaves of the axes as observed in *C. hystrix*.

The embryos in polyembryonic seeds formed a small mass at the micropylar end. These embryos were nearly globular in shape but very dissimilar in size (Oiyama and Kobayashi, 1990). Except in cultivars which rarely or never produce nucellar embryos, the zygotic embryo need not compete with one or more nucellar embryos for space within the seed. In all other cases, the result of this competition depends upon the number of embryos within the seed, their time of development, their location and vigour. Therefore, the lower the number of embryos per seed, the larger the average size of the embryos and greater probability of survival (Jackson and Gmitter, 1997). It was also noticed that the larger embryos were more likely to be stained by 2,3,5-triphenyl tetrazolium chloride than the smaller ones indicating the presence of polymorphism in viability and vigour of polyembryos in *C. hysterix*. In addition the presence of more than one axis in a seed resulted in higher germination even though some embryos were non-viable. The unstained or partially stained axes may be non-viable reflecting the low seedling production. But there is an assurance of getting one seedling from each seed. Similarly, the small embryos located near the micropylar end of the seed do not always emerge as seedlings (Andrade *et al*., 2004).

If, zygotic embryos fail to survive in all or most of the seeds, the resultant seedlings will largely be from nucellar material and therefore genetically identical to the female parent plant. Such cultivars would be referred to as “highly nucellar,” producing a large proportion of nucellar plants compared with zygotic ones (Jackson and Gmitter, 1997). Therefore, the majority of seedlings arising from polyembryonic seeds correspond to the maternal genotype and are nucellar seedlings. The nucellar seedlings thus produced were more vigourous than zygotics (Xiang and Roose, 1988). Therefore, *C. hystrix* seeds possess polyembryos in which the embryonic axis has differential vigour and viability. These differences have been decided by the embryo size, weight, number and accumulation of reserve materials. Bigger seeds with greater embryo weight and less polyembryony had vigourous seedlings. Mostly, mono-embryonic seeds produced vigourous seedlings compared with polyembryonic seeds. These viability and vigour differences influenced seedling emergence and survival. The second and later emerging seedlings in a polyembryonic axis showed poorer vigour and survival potential.

References

Aleza, P., Juarez, J., Ollitrault, P. and Navarro, L. (2010). Polyembryony in non-apomictic genotypes. *Annals of Botany*, **106**, 533-545.

Altaf, N., Murwat, E.K., Bhatti, I.A. and Mohsin Iqbal, M. (2001). Nucellar regeneration and polyembryony of citrus cultivars. *Pakistan Journal of Botany*, **33**, 211-215.

Andrade, R.M., Villegas-Monter, A., Carrillo-Castañeda, G. and García-Velázquez, A. (2004). Polyembryony and identification of Volkamerian lemon zygotic and nucellar seedlings using RAPD. *Pesquisa Agropecuária Brasileira*, **39**, 551-559.

Bacchi, O. (1943). Cytological observations in citrus III. Megasporogenesis, fertilization and polyembryony. *Botany Gazette*, **105**, 221-225.

Batygina, T.B. (2005). Embryoidogeny. In *Embryology of Flowering Plants. Terminology and Concepts, Volume 2. The Seed,* (ed. T.B. Batygina), pp. 403-409, CRC Press, London.

Batygina, T.B. and Vinogradova, G.Y. (2007). Phenomenon of polyembryony. Genetic heterogenity of seeds. *Russian Journal of Development Biology*, **38**, 126-151.

Bowman, K.D., Gmitter, F.G. and Hu, X. (1995). Relationships of seed size and shape with polyembryony and the zygotic or nucellar origin of Citrus species seedlings. *HortScience*, **30**, 1279-1282.

Cameron, J.W. and Garber, M. (1968). Identical twin-hybrids of Citrus × Poncirus from strictly sexual seed parents. *American Journal of Botany*, **55**, 199-205.
Ellis, R.H. and Roberts, E.H. (1981). The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology*, **9**, 373-409.
Jackson, L.K. and Gmitter, F.G. (1997). *Seed Development in Citrus* <http://irrec.ifas.ufl.edu/ flcitrus/pdfs/ short_course_and_workshop/citrus_flowering_97/Jackson-Seed_ Development.pdf>
Koltunow, A.M. (1993). Apomixis embryo sacs and embryos formed without meiosis or fertilization in ovules. *The Plant Cell*, **5**, 1425-1437.
Koltunow, A.M., Soltys, K., Nito, N. and McClure, S. (1995). Anther, ovule, seed and nucellar embryo development in *Citrus sinensis* (L.) cv. Valencia. *Canadian Journal of Botany*, **73**, 1567-1582.
Moore, R.P. (1972). Effects of mechanical injuries on viability. In *Viability of Seeds* (ed. E.H. Roberts), pp. 94-113, Syracuse University Press, New York.
Oiyama, I. and Kobayashi, S. (1990). Polyembryony in undeveloped monoembryonic diploid seeds crossed with a citrus tetraploid. *HortScience*, **25**, 1276-1277.
Ozsan, M. and Cameron, J.W. (1963). Artificial culture of small citrus embryos and evidence against nucellar embryony in highly zygotic varieties. *Proceedings of American Society of Horticultural Science*, **87**, 210-216.
Soares Filho, W. dos S., Lee, L.M. and da Cunha Sobrinho, A.P. (1995). Influence of pollinators on polyembryony in *Citrus*. *Acta Horticulturae*, **403**, 256-265.
Wakana, A. and Uemoto, S. (1988). Adventive embryogenesis in citrus (Rutaceae) II. Post fertilization development. *American Journal of Botany*, **75**, 1033-1047.
Xiang, C. and Roose, M.L. (1988). Frequency and characteristics of nucellar and zygotic seedlings in 12 citrus rootstocks. *Scientia Horticulture*, **37**, 47-59.