Apomictic seed development in *Ensete superbum* induced by pollen of wild banana sp. *Musa balbisiana*

Intergeneric hybridization, especially in vegetatively propagated crop species involving wild relatives, is a common method to introgress desirable genes into the crop species. The family Musaceae has two genera, *Musa* and *Ensete*[1]. An intergeneric cross was attempted between *Ensete superbum* ($2n = 18$) and *Musa balbisiana* ($2n = 22$) to transfer some characters related to abiotic and biotic stress tolerance[2]. Generally, it is difficult to produce intergeneric hybrids due to various pre- and post-fertilization barriers. In *Musa*, pollination, seed set and germination are extremely variable and thus relatively difficult to obtain hybrids.

One hundred *Ensete* seeds were collected from the Western Ghats region and sown in the nursery at the Indian Institute of Horticultural Research, Bangalore. One seedling germinated and flowered after three years. *M. balbisiana* was available in our banana germplasm collections. A cross was made between *E. superbum* ($2n = 18$) and *M. balbisiana* ($2n = 22$), as female and male parents respectively. The *Ensete* (Figure 1 *a*) bunch had 19 hands with 248 flowers (14–16 female flowers per hand). The entire bunch was bagged to avoid any cross-pollination. In the first nine hands, 108 flowers were not pollinated, and in the next ten hands, 140 flowers were pollinated with *M. balbisiana* pollen. We observed seed set only in the hands where pollination was done using *M. balbisiana* pollen. Each pollinated fruit consisted of more than 200 seeds. Nearly 5000 good seeds were collected, washed and stored. Seed germination in the nursery with regular potting mixture was not successful. Embryo culture technique[3] was used to germinate the embryos *in vitro*.

It was observed that about 5% of seeds were without embryos or had underdeveloped embryos. Among the five different media used for the germination of embryos, media (M1), i.e. MS without any hormones showed the highest percentage germination (72) with an average germination time of 5 days. Germination reduced when meristematic region of the embryo was fully embedded inside the medium. Embryos with the haustorium end touching the media resulted in good germination (95%). Fully developed plants were transferred to the nursery for hardening (Figures 1 *b*). During embryo culture, unsoaked seeds did not germinate. Water soaking might have helped in germination by washing away dormancy inducing chemicals. Similar observation was made by Afele and De Langhe[4], in case of *M. balbisiana* seeds.

Cytological studies to count the number of chromosomes were done using karyotyping techniques in both the parents and four putative 'hybrid seedlings'. The chromosome number of *E. superbum* and *M. balbisiana* was found to be $2n = 18$ and $2n = 22$ respectively (Figure 2 *a* and *b*). All the four seedlings of putative hybrids observed had $2n = 18$ chromosomes (Figure 2 *c*). This indicates that the seedlings might have inherited their diploid set of chromosomes from their female parent, *E. superbum* with $2n = 18$, with no contribution from the male parent, *M. balbisiana*. This was further confirmed by molecular studies.

In order to determine the hybridity of the seedlings, ITS markers were employed. Total genomic DNA was extracted following the modified CTAB method[5] from the young leaves of parents and 18 *in vitro* germinated F1 seedlings. Two primers, ITS-L (ref. 6) and ITS-4 (ref. 7), were used for PCR amplification of the ITS region. The PCR products were digested using *Rsa*I and *Taq*I (Fermentas Inc.). The digested PCR fragments were separated by electrophoresis on 2% agarose gel. Fragment sizes were estimated by comparison with 100 basepair ladder.

Amplification of ITS region using ITS-L–ITS-4 primers produced a 700 bp

Figure 1. ***a***, Female parent, *Ensete*; ***b***, F1 seedling resembling female parent.

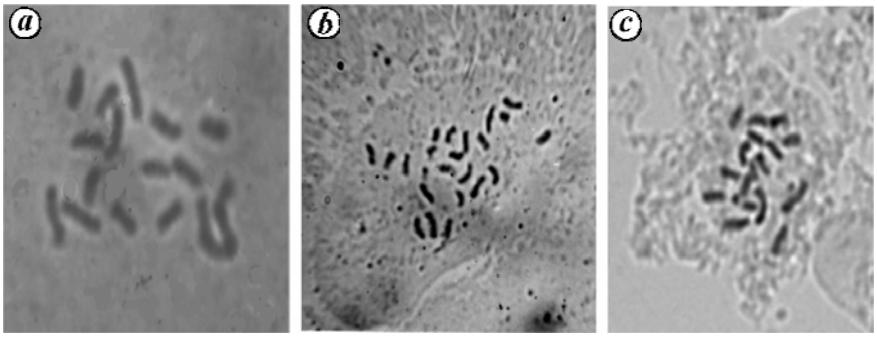

Figure 2. Karyotype analysis in parents and F1 seedling: ***a***, *Ensete superbum* chromosome no. $2n = 18$; ***b***, *Musa balbisiana* chromosome no. $2n = 22$; ***c***, *E. superbum* × *M. balbisiana* chromosome no. $2n = 18$.

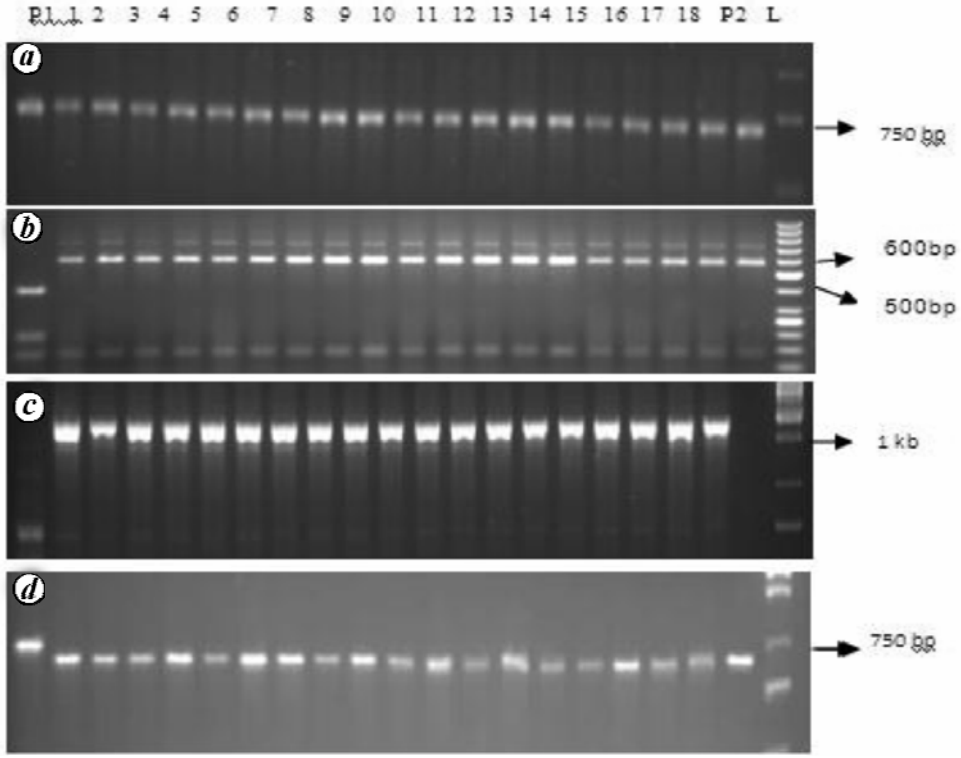

Figure 3. Gel photograph showing amplification and restriction digestion pattern for the ITS region. ***a***, Amplification of ITS region using ITS-L and ITS-4 primer produced 750 bp fragment for both parents P1 (*M. balbisiana*), P2 (*E. superbum*) and F1 seedlings from lanes 1 to 18. ***b***, Restriction digestion pattern of amplified ITS region using *Rsa*1 enzyme; ***c***, Amplified products of chloroplast marker (rpS14-cob) for both parents P1 (*M. balbisiana*), P2 (*E. superbum*), and F1 seedlings from lanes 1 to 18. ***d***, Amplified products of mitochondrial marker (5srRNA–18srRNA).

fragment (Figure 3 *a*) in both the parents and all the seedlings. Both restriction enzymes used in this study, *Rsa*I and *Taq*I, produced a consistent polymorphic banding pattern between *M. balbisiana* and *E. superbum*. The *Rsa*I digest produced three fragments of 340, 190 and 120 bp for *M. balbisiana*, while three fragments of 650, 550 and 120 bp were observed for *E. superbum* (Figure 3 *b*). The *Taq*I digest produced three fragments of 180, 110 and 50 bp for *M. balbisiana*, while three fragments of 250, 180 and 70 bp were observed in *E. superbum*. When the PCR product from F1 seedlings was digested with these enzymes, all of them showed restriction-banding pattern similar to that of female parent, *E. superbum*, indicating there was no fertilization.

The maternal transmission of chloroplast DNA and paternal transmission of the mitochondrial DNA help in studying the maternal and paternal lineage of clones in banana. Hence organellar genome distribution among the progenies from the parents, *E. superbum* and *M. balbisiana* was determined using the chloroplast and mitochondria-specific primers. Five mitochondria primers and 30 chloroplast primers were screened initially with parents[8].

The chloroplast (rpS14-cob) and mitochondrial (5srRNA–18srRNA) markers, which showed consistent polymorphic bands for the parents, *M. balbisiana* and *E. superbum* were used to analyse the F1 seedlings. Both the chloroplast and mitochondria markers showed a banding pattern similar to that of *E. superbum* in all the seedlings (Figure 3 *c* and *d*). Earlier studies have clearly shown maternal inheritance of chloroplast genome and paternal inheritance of mitochondrial genome in *Musa acuminata*[9]. Here seedlings inherited both mitochondrial and chloroplast genes from maternal parent, *E. superbum*. This shows that there was no fertilization of gametes involving *M. balbisiana*.

In cultivated banana, fruit development is parthenocarpic; pollination in wild bananas and *Ensete* is by insects and bats. There are reports of unreduced female and male gamete formation in *Musa* spp. and cultivars[10]. The flowering occurs in phases (female followed by male). The natural *Ensete* population seems to be uniform and stable. Earlier studies on ovule development have shown that the *Ensete* ovules were significantly large in size compared to the *Musa* spp. Also, the nucellus had higher number of cells[11]. However, hybridization and fusion of male and female gametes has not been studied.

Both the above studies, i.e. cytological and molecular, clearly indicate that the seed might have been developed apomictically. In apomictic reproduction, the embryo develops autonomously from an unreduced cell having the same set of maternal chromosomes and giving rise to plants that are clones of the mother plant known as gametophytic apomixis[12]. This mode of reproduction occurs in about 35 families of angiosperms, including Liliaceae[13] and Poaceae[14].

Such a phenomenon has not been observed earlier in Musaceae. It is well known that most members of Poaceae

have a strong and highly evolved background for apomixis[15], and it is likely that Musaceae might have acquired its apomictic character from its neighbour, Poaceae[16].

In an earlier unpublished study it was found that there was no seed set when *M. acuminata* pollen was used on *E. superbum* (pers. commun.). Also there was no seed set when no pollen was used for pollination, as observed in this study. Seeds were formed only when *M. balbisiana* pollen was applied. This supports the fact that the kind of pollen applied in crosses influences the balance between apomictic and sexual reproduction in facultative apomicts[17]. This is further supported by a report of Sobolev *et al.*[18], who observed apomictic pod formation in pea under the influence of pollen from vetch, lathyrus and lentil.

As the seedlings are diploid and resemble the maternal parent, there is a possibility that eggs are unreduced. This may be a characteristic feature of *Ensete*, which needs to be examined. Here in this study, pollen from *M. balbisiana* might have provided the right kind of stimulus for apomixis. However, studies on pollen tube growth and early stages of embryo development would confirm this hypothesis. The present studies were carried out using one plant of *Ensete*. There is a need to further explore possibilities like whether *Ensete* naturally produces apomictic seeds or pollination is necessary for seed development (either by its own or others like *M. balbisiana* pollen). Although our attempt to produce a true hybrid between the two wild genera of Musaceae has not been successful, here we report the possibility of apomictic embryo formation in Musaceae. This knowledge reveals the fact that intergeneric hybridization may not be a possibility in conventional banana breeding.

1. Chessman, E. E., *Kew Bull.*, 1947, **2**, 97–117.
2. Vuylsteke, D. and Swennen, R., In Proceedings of a Workshop on Biotechnology Applications for Banana and Plantain Improvement, San Jose, Costa Rica, 1993, pp. 169–176.
3. Diro, M. and Van Staden, J., *S. Afr. J. Bot*., 2004, **70**, 635–639.
4. Afele, J. C. and De Langhe, E., *Plant Cell Tissue Organ Cult*., 1991, **27**, 33–36.
5. Ravishankar, K. V., Anand, L. and Dinesh, M. R., *J. Hortic. Sci. Biotechnol.*, 2000, **75**, 198–201.
6. Nwakanma, D. C., Pillay, M., Okoli, B. E. and Tenkouano, A., *Theor Appl. Genet*., 2003, **108**, 154–159.
7. White, T. M., Bruns, T., Lee, S. and Taylor, J., In *PCR Protocols: A Guide to Methods and Applications* (eds Innis, M. A. *et al.*), Academic Press, San Diego, CA, 1990, pp. 315–321.
8. Demesure, B., Sodzi, N. and Petit, R. J., *Mol. Ecol*., 1995, **4**, 129–131.
9. Faure, S., Noyer, J. L., Carreel, F., Horry, J. P., Bakry, F. and Lanaud, C., *Curr. Genet*., 1994, **25**, 265–269.
10. Simmonds, N. W., In *The Evolution of the Bananas*. Longman Publications, London, 1962.
11. Fortescue, J. A. and Turner, D. W., *Sci. Hortic.*, 2005, **104**, 463–478.
12. Carneiro, V. T. C., Dusi, D. M. A. and Ortiz, J. P. A., In *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (ed. Teixeira da Silva, J. A.), Global Science Books, Isleworth, UK, 2006, vol. I, 1st edn, pp. 564–571.
13. Proscevičius, J., Rančelienė, V. and Dambrauskaitė, D., *Biologia*, 2007, **53**, 8–12.
14. Dewald, C. L. and Kindiger, B. K., *Am. J. Bot*., 1998, **85**, 1237–1242.
15. Asker, S. E. and Jerling, L., In *Apomixis in Plants*, CRC Press, Boca Raton, 1992, p. 298.
16. Lescot, M. *et al.*, *BMC Genomics*, 2008, **9**, 58.
17. Ozias-Akins, P. and van Dijk, P. J., *Annu. Rev. Genet*., 2007, **41**, 509–537.
18. Sobolev, N. A., Adamchuk, G. K. and Zhila, E. D., In *Apomixis in Pea*. Amerind Publishing Co, New Delhi, 1976, pp. 221–227.

ACKNOWLEDGEMENT. This work was supported through the 'ICAR Network Project on Transgenic in Crops: Functional genomics of Fusarium wilt and drought tolerance in banana' from the Indian Council of Agricultural Research, New Delhi.

Received 8 October 2010; revised accepted 21 July 2011

KUNDAPURA V. RAVISHANKAR[1,*]
REKHA AJITHA-KUMAR[2]
MALARVIZHI MATHIAZHAGAN[1]
DASANNANA-MALIGE S. AMBIKA[2]

[1]*Division of Biotechnology and*
[2]*Division of Fruit Crops,*
Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore 560 089, India
**For correspondence.*
e-mail: kv_ravishankar@yahoo.co.in