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Performance evaluation of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules

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Abstract

This experiment was carried out at the High Rainfall Station of the International Institute of Tropical Agriculture (IITA) in Onne, Rivers State, Nigeria, to investigate if secondary triploid plantains established using *in-vitro* excised zygotic embryo propagules and tissue culture will differ significantly from those established by using *ex-vitro* propagules such as seeds obtained from open or hand pollinated parents. Seeds of secondary triploid plantain hybrids were obtained by crossing tetraploid female hybrids TMP4x 2796-5 and TMP4x 4698-1 by hand pollination with pollen from known diploid males TMP2x 1297-3 and TMP2x 1448-1. Another set of these female hybrids were exposed to open pollination. At maturity, seeds from ripened mature fruits from both methods of pollination had their zygotic embryos extracted and cultured *in-vitro*. Another set of seeds were grown *ex-vitro* in the soil. Both *in-vitro* and *ex-vitro* derived propagules obtained from open and hand pollinated parents were raised in the tissue culture laboratory and the nursery respectively. Three replications of the 12 treatments of eight-week-old secondary triploids were planted in the field at 3m x 2m spacing using an RCB (randomized complete block) design. Data collected were: number of days to 50% flowering (DTF), time to fruit filling (TFF), plant height and number of leaves at 50% flowering, bunch weight, number of hands/bunches, fingers/ bunch and total yield. Collected data were analysed using ANOVA in RCB design at (P = 0.05) while means were compared with Duncan's Multiple Range Test at (*P* = 0.05). Inconsistent individual genotypically significant differences were found in some phenological and vegetative characters, yield constituents and total yield between *ex-vitro* and *in-vitro* derived secondary triploids indicating no definitive pattern in responses of the secondary triploids. However, only days to flowering (DTF) showed consistent significant notable differences out of all these traits. Generally, the *ex-vitro* derived secondary triploids flowered significantly later than *in-vitro* derived ones. The secondary triploids from open pollinated parental crosses flowered significantly earlier than those obtained from the hand pollinated parental crosses.

Keywords: Embryo rescue; zygotic embryo; in-vitro, ex-vitro; phenological traits; propagules

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INTRODUCTION

The *Musa* spp., of edible bananas and plantains are one of the most valuable and prominent food crops in the world. Their importance is however threatened by inherent and external biotic and abiotic factors that cause severe economic and productivity losses especially in the sub-Saharan tropical region (Wairegi *et al*., 2010; Fortescue and Turner 2011; Hippolyte *et al*., 2012; Brown *et al*., 2017; Pua *et al*., 2019; Waniale *et al*., 2021). Like plantains and bananas before now, some plants and other synthetic varieties could not be readily propagated by seeds either because they did not readily produce seeds, produced seeds that were unable to grow to maturity or because the seeds they produced were difficult to germinate, etc. (Chin 1995; Ortiz and Vuylsteke,1995; Graven *et al*.,1996; Dumpe and Wokoma, 2003). One of the approaches to solving these problems is by priming of seeds for those that produce seeds thus breaking the barriers that inhibit germination and improving their capacity to germinate (Wilson and Tenkouano, 2019a; Wilson 2019a. Wilson, 2022). Another approach is to use other parts of the plant like tubers, suckers, vines, stem cuttings and root cuttings, axillary meristem, shoot tip /apical meristems, male flowers, etc., for their propagation (Vuylsteke, 1989; Vuylsteke, *et al*., 1990; Swennen and Vuylsteke 1993; Darvari *et al*., 2010; Wilson, 2019b; Wilson and Tenkouano, 2019b; Wilson and Tenkouano, 2020; Adjei *et al*., 2021). For those that do not readily produce viable seeds however, the approach as pointed out by Ortiz (2013) has been through embryo rescue of zygotic embryos or somatic embryos and *in-vitro* tissue culture techniques in the laboratory to raise plantlets (Swennen and Vuylsteke, 1993). The use of embryo rescue technique no doubt dramatically improved availability of propagules for *Musa* breeding and today, use of embryo rescue and *in-vitro* tissue culture techniques has become standard practice for production of many of the widely accepted secondary triploid varieties of *Musa.* However, hybrid seed production and germination in *Musa* for purposes of breeding which limited propagation exclusively to embryo rescue and *in-vitro* tissue culture techniques is no longer as difficult and challenging as it was decades ago, thanks to several studies conducted by many researchers over the years especially in, but not limited to, institutions such as Embrapa (Empresa Brasileira de Pesquisa Agropecuária) in Brazil; FHIA (Fundación Hondureňa de Investigaçion Agricŏla) in Honduras; and IITA (International Institute of Tropical Agriculture) in Nigeria among others (Chin,1995; Vuylsteke and Ortiz, 1996; Tenkouano *et al*., 2011; Amorim *et al*., 2013; Wilson and Tenkouano, 2019a, Wilson *et al*., 2020). More research is needed to investigate the overall performance of secondary triploid plantains grown from propagules derived by zygotic embryo rescue and *in-vitro* culture and those raised by seeds grown *ex-vitro* in soil whether hand or open pollinated. Interestingly even though questions of more and better quality seed production by open pollination than by hand pollination methods seem to have been satisfactorily settled over the years (Ortiz and Vuylsteke, 1995; Ortiz and Crouch, 1997; Ortiz 2013) there are as yet no deliberate concerted or speedy attempts to move away from hand pollination to open pollination for seed production in *Musa* even when it has been reported that open and hand pollination did not show significant or substantial variations in yield constituents nor even in yields of *Musa* (Wilson and Tenkouano, 2019c). This is quite surprising given the huge cost implications involved in hand pollination relative to open pollination processes and the additional high financial overheads involved in pursuing the embryo rescue *in-vitro* culture approach of propagule production and setting up a tissue culture facility (Jacobsen *et al*., 2019) compared to the *ex-vitro* seed approach despite strong advocacy pointing out the benefits of *ex-vitro* seed use over the years (Tenkouano, 2000; Van den houwe *et al.,* 2020). Perhaps a definitive study about whether phenology and yield of secondary triploid plantains grown by *in-vitro* excised zygotic embryos relative to those grown by seeds *ex-vitro* can help researchers take a more definitive decision. This experiment was therefore set up to find out if secondary triploid plantains established using *in-vitro* excised zygotic embryo propagules and tissue culture will differ significantly from those established by using *ex-vitro* propagules such as seeds obtained from open or hand pollinated parents.

MATERIALS AND METHODS

Study area and geophysical characteristics

This research was carried out consecutively at the plant tissue culture laboratory, the nursery, the green house and open field at Onne, IITA (International Institute of Tropical Agriculture) High Rainfall Station, Rivers State, in the Niger

Delta region of Nigeria (4°51'N, 7° 03'E, 10m above sea level). The rainfall exhibits a monomodal pattern with an annual mean of 2400mm, spread across ten months usually from February to December. Mean relative humidity ranges from 78% - 89% and is high all year round. Mean annual temperatures are between 25^0C minimum and 27^0C maximum (Ortiz *et al*., 1997). The soil is acidic at a pH of 4.3 - 4.6; a Typic Paleudult and derived highly leached Ultisol of the Niger Delta with organic matter content of 1.85%, 0.28 me/100g potassium, 60 mg kg-1 phosphorus, 0.36-me/100g magnesium and 0.2 mmol kg-1 manganese and a low total nitrogen of 0.18% (Wilson *et al*., 2020).

Experimental procedure

Crossing of parents to obtain propagules

The propagules of the secondary triploid plantain hybrids were obtained by bagging in the field, the female inflorescence of two tetraploid female hybrids TMP4x 2796-5 and TMP4x 4698-1 at anthesis and crossing them by hand pollination in the early hours of the morning before 10am with pollen from two known diploid males TMP2x 1297-3 and TMP2x 1448-1. The inflorescences were bagged again immediately after hand pollination to prevent contamination of pollen from other males thus ensuring that only pollen from the identified males pollinated these females. Another set of the same tetraploid female hybrids were left to open pollination, in which the pollen was from unknown males. At maturity, bunches from both open and hand pollinated females were treated with ethylene for a four-day period to ripen them and the seeds removed, washed thoroughly in tap water and air-dried.

Derivation of propagules

***In-vitro* derived propagules obtained by embryo rescue of zygotic embryos**

The seeds obtained from both tetraploid female parents that were open and hand pollinated were treated as described by Wilson and Tenkouano (2019a) using the method of Vuylsteke *et al*., (1990) for rescue of zygotic embryos followed by tissue culture. Daily inspection of the cultures was carried out and germinated seedlings were removed to acclimatize in the greenhouse.

Ex-vitro derived propagules planted in soil

Soil treated with carbendazim (Bio supercarb, at 0.052M) was placed in plastic pots of size 16cm by 13cm by 4.9cm which were then perforated and filled to three-quarter level. Three days after soil treatment, 10 seeds obtained from each of the open and hand pollinated tetraploid female parents were planted in each pot and raised inside the nursery. The potted seeds were watered as necessary and the pots were examined every week for signs of germination. Any seed with its plumule emerging 1cm above the soil was deemed to have germinated.

Treatment applications and experimental design

The applied treatments comprised 12 under listed hand and open pollinated *in-vitro* and *ex-vitro* derived secondary triploids (ST) as shown below.

Treatments	Parental crosses and methods of pollination*	Derived propagules
ST 1	= TMP4x 2796 5 x TMP2x 1297-3 (Hand Pollinated)	*In-vitro*
ST 2	= TMP4x 2796 5 x TMP2x 1297-3 (Hand Pollinated)	*Ex-vitro*
ST 3	= TMP4x 2796 5 x TMP2x 1448-1 (Hand Pollinated)	*In-vitro*
ST 4	= TMP4x 2796 5 x TMP2x 1448-1 (Hand Pollinated)	*Ex-vitro*
ST 5	= TMP4x 2796 5 Open pollinated†	*In-vitro*
ST 6	= TMP4x 2796 5 Open pollinated†	*Ex-vitro*
ST 7	= TMP4x 4698 1 x TMP2x 1297-3 (Hand Pollinated)	*In-vitro*
ST 8	= TMP4x 4698 1 x TMP2x 1297-3 (Hand Pollinated)	*Ex-vitro*
ST 9	= TMP4x 4698 1 x TMP2x 1448-1 (Hand Pollinated)	*In-vitro*
ST 10	= TMP4x 4698 1 x TMP2x 1448-1 (Hand Pollinated)	*Ex-vitro*
ST 11	= TMP4x 4698 1 Open pollinated†	*In-vitro*
ST 12	= TMP4x 4698 1 Open pollinated†	*Ex-vitro*

***** *Total number of crosses evaluated from both pollination methods = 228,* **†** *unknown male / pollen source*

Eight-week-old seedlings from the open and hand pollinated secondary triploid hybrids obtained by *in-vitro* and *ex-vitro* derived propagules were transplanted to the field at 3m x 2m spacing with three replications of the 12 treatments. The experimental design was an RCB (Randomized Complete Block) design. Field maintenance and cultural procedures were as described by Wilson and Tenkouano (2019c).

Collection and statistical analyses of data

The data for selected phenological and vegetative traits were collected over two crop cycles as follows: (i) number of days to 50% flowering (DTF) (ii) time to fruit filling (TTF), (iii) plant height at 50% flowering (iv) total number of fully formed leaves at 50% flowering. Those for yield and yield constituents over the same period were collected as follows: (v) weight of bunch (vi) total number of hands/ bunch (vii) total fingers/ bunch (viii) and total yield. All the data collected were evaluated by analysis of variance (ANOVA) using RCBD (**Gomez and Gomez, 1984**) at a significance level of 5% (P=0.05) for the F-test. Means were compared by using the DMRT (Duncan's Multiple Range Test) at (P=0.05) whenever the F-test was significant.

RESULTS

Phenological and vegetative traits

Days to 50% flowering

The *ex-vitro* derived secondary triploid from the open pollinated cross between the female TMP4x 4698-1 and an unknown male (ST 12) was first to flower in 273days (Fig. 1a). It flowered significantly earlier (P = 0.05) than all other secondary triploids. The next to flower 12 days later was the *in-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1448-1 (ST 3) followed a day later by (ST 5) the *in-vitro* derived secondary triploid from the open pollinated female parent TMP4x 2796-5. The *ex-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1448-1 (ST 4) flowered last. Significant differences were also found between *in-vitro* and *ex-vitro* derived secondary triploids in number of days to flowering (DTF). Generally, the *ex-vitro* derived secondary triploids flowered 43days later than *in-vitro* derived ones. Moreover, the secondary triploids from open pollinated parental crosses flowered 36days earlier than those obtained from the hand pollinated parental crosses. The genotype of female parents in the hand pollinated crosses that produced the secondary triploids did not significantly affect DTF. However, in the open pollinated females, the secondary triploids from TMP4x 4698-1 flowered significantly earlier than those from TMP4x 2796-5. Moreover, there was a significant effect of male genotype or pollen source on DTF. The open pollinated secondary triploids of unknown pollen source had DTF earlier than both of the known male parents and they flowered 38days earlier than with male parent TMP2x 1297-3 and 34days earlier than with male parent TMP2x 1448-1.

Time to fruit filling

The *ex-vitro* derived secondary triploid from the open pollinated parental cross between the female TMP4x 4698-1 and an unknown male (ST 12) had the shortest time to fruit filling (TFF) in 100 days (Fig. 1b). It achieved TFF significantly earlier (P = 0.05) than seven other secondary triploids. The next TFF, 10 days later, was the *in-vitro* derived secondary triploid from the open pollinated female parent TMP4x 2796-5 (ST 5), followed by the *in-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1297-3 (ST 1). No significant differences in TFF were observed between *ex-vitro* and *in-vitro* derived secondary triploids. Overall, no significant differences were found between *in-vitro* and *ex-vitro* derived secondary triploids from open and hand pollinated parents. Moreover, genotypes of female and male parents did not show significant differences with respect to TFF.

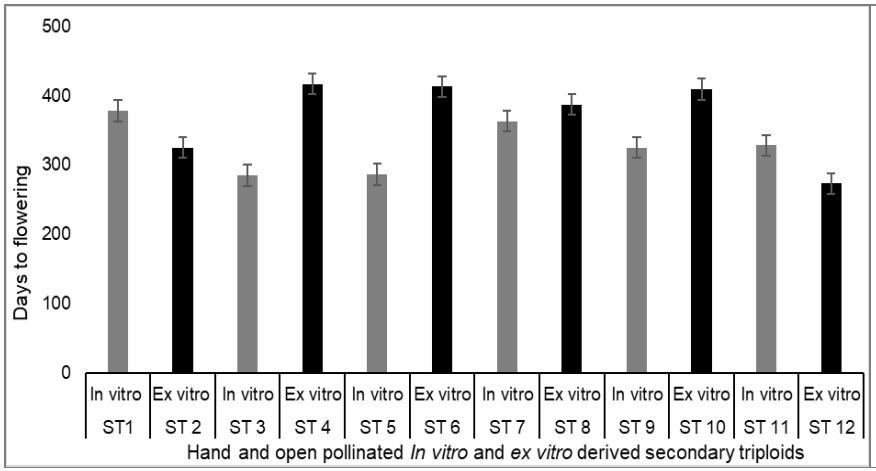

Fig. 1a. Days to 50% flowering of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

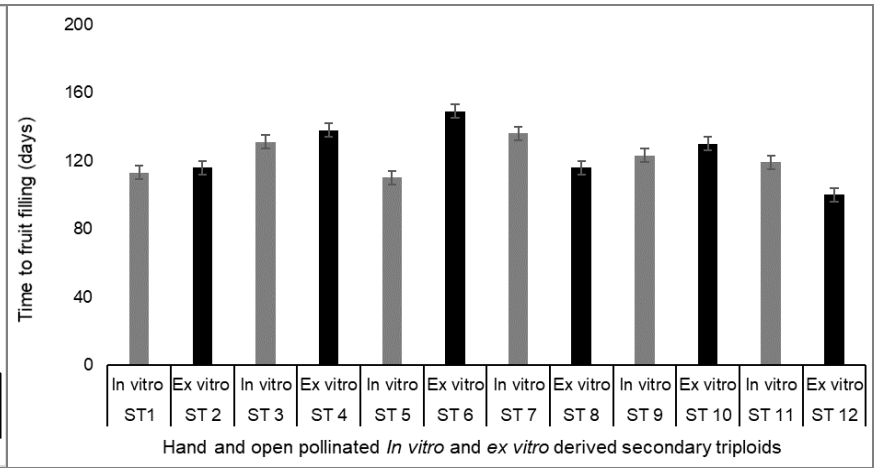

Fig 1b. Time to fruit filling of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

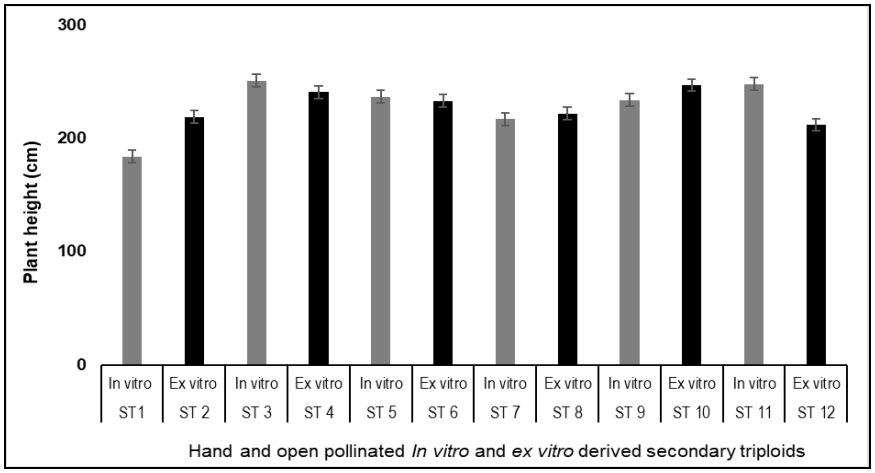

Fig 1c. Plant height of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

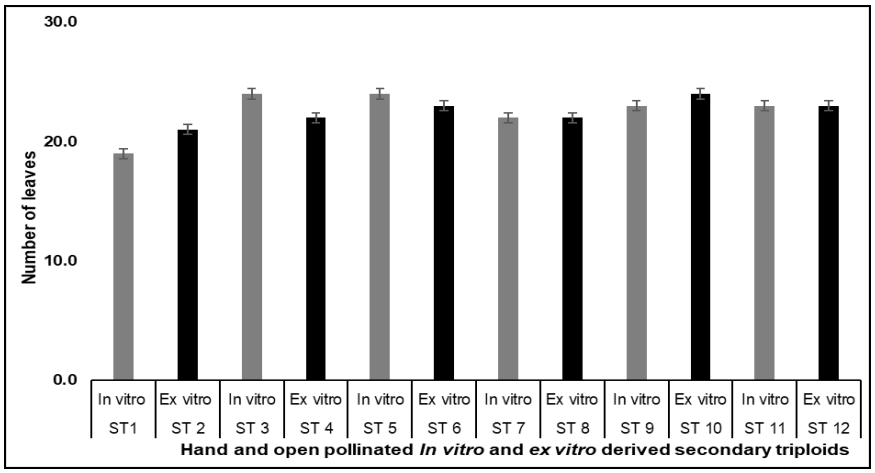

Fig 1d. Number of leaves of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

Plant height

The height of secondary triploids is presented in Fig 1c. The *in-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1297-3 (ST 1) had the shortest plants at 184cm and was significantly shorter (*P* = 0.05) than seven others. The next shortest was the *ex-vitro* derived secondary triploid from the open pollinated female TMP4x 4698-1 (ST 12) at 212cm. However, overall, *in-vitro* and *ex-vitro* derived secondary triploids did not differ significantly whether from open and hand pollinated parents. In addition, genotypes of female and male parents did not show significant differences with respect to plant height.

Number of leaves

The *in-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1297-3 (ST 1) had 19 leaves; the fewest leaves at maturity and this was significantly fewer (*P* = 0.05) than leaves of ten other triploids (Fig 1d). Comparing *in-vitro* and *ex-vitro* derived secondary triploids, total leaves did not differ significantly. The genotypes of female and male parents of the secondary triploids did not significantly affect total leaves present neither did the method of pollination of the parents

Yield constituents and total yield

Weight of bunches

The hand pollinated *ex-vitro* derived secondary triploid from the parental cross TMP4x 4698-1 x TMP2x 1448-1 (ST 10) had significantly higher bunch weight of 5.2kg than only five of the other secondary triploids (Fig 2a). Both (ST 1), hand pollinated TMP4x 2796-5 x TMP2x 1297-3 *in-vitro* derived secondary triploid and (ST 12) the TMP4x 4698-1 open pollinated *ex-vitro* derived secondary triploid with pollen from unknown male had the lowest bunch weights of 2.7 kg. When compared generally, significant differences were not found in bunch weights of *in-vitro* and *ex-vitro* derived secondary triploids. Likewise, bunch weights of secondary triploids from hand and open pollinated parents did not differ significantly. Genotypes of female and male parents did not significantly affect bunch weight of the secondary triploids.

Total number of hands/bunches

Both the *ex-vitro* derived secondary triploid from the hand pollinated parental cross TMP4x 2796-5 x TMP2x 1448-1 (ST 4) and the *in-vitro* derived secondary triploid from the parental cross TMP4x 4698-1 x TMP2x 1448-1 (ST 9) each had the highest total hands/bunch of 7.5 (Fig 2b). They had significantly higher (*P* = 0.05) total hands/ bunch than three other secondary triploids but did not differ significantly from the other seven. Overall, there were no other significant differences.

Total number of fingers/bunches

Both open pollinated *ex-vitro* and *in-vitro* derived secondary triploids from the cross between the female parent TMP4x 2796-5 and unknown male pollen parents (ST 4 and ST 5) gave highest fingers/ bunch of 118.8 and 118.3 respectively (Fig 2c). However, they had significantly higher (*P*= 0.05) number of fingers than only four of the other secondary triploids. Other parameters measured did not significantly differ.

Total yield

The *ex-vitro* derived hand pollinated secondary triploid from the parental cross TMP4x 2796-5 x TMP2x 1448-1 (ST 4) had the highest yield of 12.9kg and this was significantly higher than yields of nine other secondary triploids (Fig 2d). On average, yields of *in-vitro* and *ex-vitro* derived secondary hybrids did not differ significantly. Other parameters measured did not significantly affect total yields.

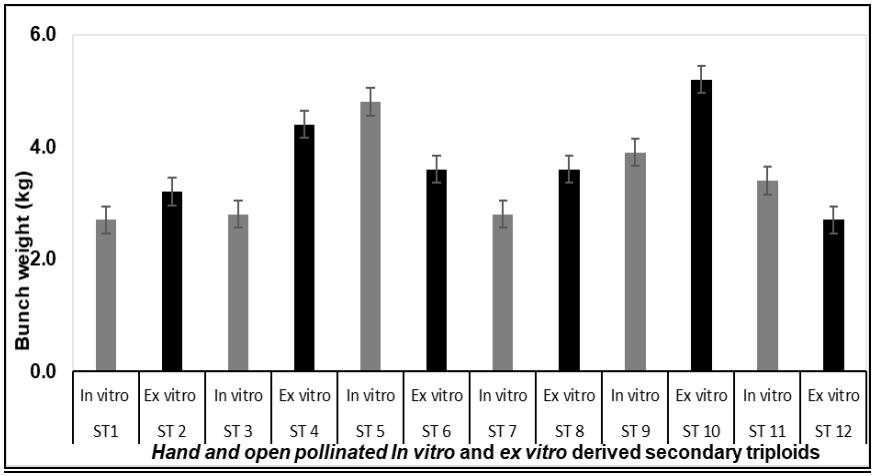

Fig. 2a. Bunch weight of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

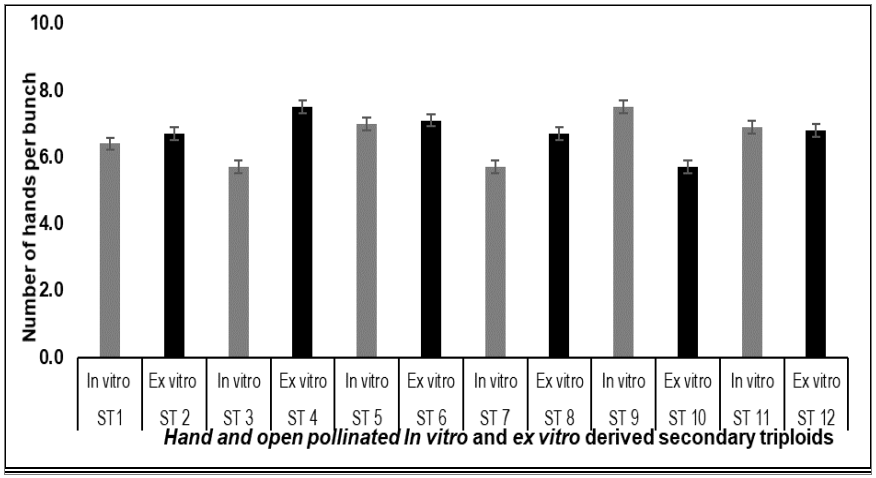

Fig. 2b. Number of hands/ bunch of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

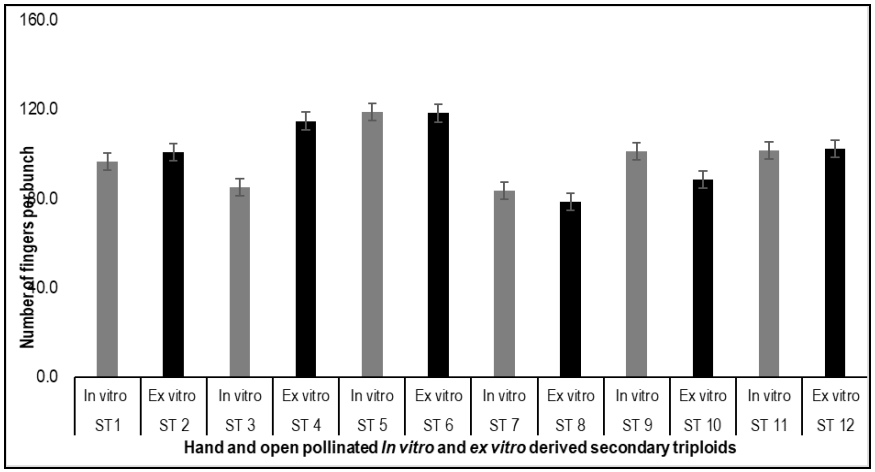

Fig. 2c. Number of fingers/ bunch of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

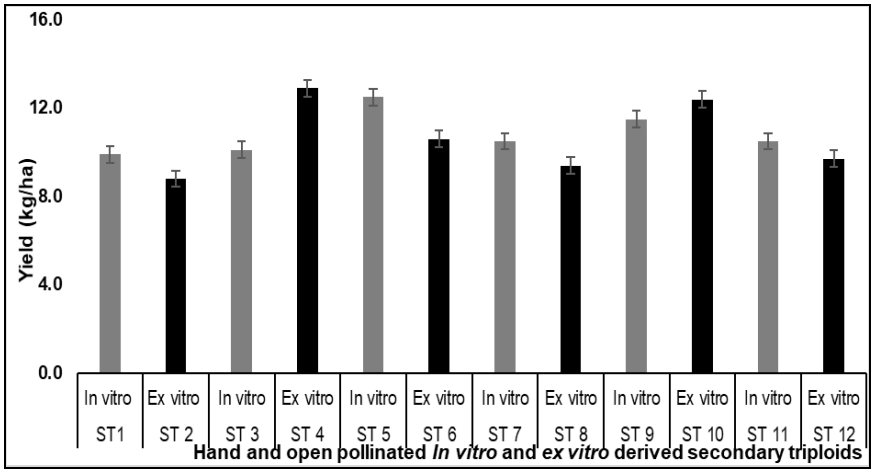

Fig 2d. Yield of secondary triploid *Musa* hybrids from *in-vitro* and *ex-vitro* derived propagules of open and hand pollinated parents

DISCUSSION

The labour, time and stress not to mention the cost expended in excising embryos from the hard seeds of *Musa* for *in-vitro* culture /germination are often glossed over or rarely mentioned. Although generally the performance of *in-vitro* and *ex-vitro* derived secondary triploids did not differ significantly with respect to TFF, vegetative characters, yield constituents (weight of Bunches, total number of hands/bunch and total number of fingers/bunch) and total yields, individual significant differences were found among the secondary triploids regarding some of these characters. These individual significant differences were inconsistent in the sense that no secondary triploid showed significant differences for more than one character. These individual significant differences could be due to genotypic differences in the secondary triploids (Naranjo *et al*., 2016). Such differences are expected as several of these traits are under polygenic control and segregation is expected because of the heterozygous nature of their parents (Damodaran 2004). Genotypic differences in the same species have been widely reported and they constitute an important factor in most biological activities. Therefore, the individual significant differences exhibited by *ex-vitro* and *in-vitro* derived secondary triploids are not surprising (Wilson and Tenkouano, 2019c; Wilson and Tenkouano, 2020). Rieger (2006) stated that a minimum of 8-10 functional leaves are enough for proper bunch maturity and in this study none of the plants had less than 19 functional leaves. Overall, the i*n-vitro* derived secondary triploids did not manifest superior performance compared to *ex-vitro* derived secondary triploids. A similar trend was reported by Vuylsteke and Ortiz, (1996) when comparing *in-vitro* and sucker propagation. However, with respect to a phenological trait like DTF, the *in-vitro* and *ex-vitro* derived secondary triploids did not only show significant differences individually, but they also revealed notable, consistent and significant overall differences in their DTF. The secondary triploids derived *in-vitro* and cultured in readily available nutrient media coupled with growth hormones probably had a growth stimulating advantage that enabled them grow faster and more vigorously in the early stages of growth, thus allowing them to achieve flowering significantly earlier than the *ex-vitro* derived secondary triploids that did not have such advantages (Arias, 1993; Akhilesh *et al*., 2017). These advantages however apparently did not generally extend beyond exhibiting earliness in flowering for most of these plants. It is also possible that some of the secondary triploids that are photoperiod sensitive could also have been induced to flower early hence shorter DTF (Parrot, 1993). Whereas the genotype of female parents did not significantly affect DTF, the genotype of the male parents appeared to have played a role in determining DTF. Overall, secondary triploids from the open pollinated parents flowered significantly earlier than those from hand pollinated parents perhaps because of the effect of the male parents contributing pollen from unknown source(s) suggesting a male genotype effect for early flowering. Nyine and Pillay (2007) had suggested earlier that germination potential of pollen in *Musa* could be genetically inherited and controlled from male parents.

CONCLUSION

This study showed that there were individual but inconsistent significant genotypic differences in phenological and vegetative traits, as well as in yield constituents and total yield between *ex-vitro* derived and *in-vitro* derived secondary triploid plantain hybrids. However only days to flowering exhibited consistent significant and notable differences between *ex-vitro* derived and *in-vitro* derived secondary triploid plantain hybrids. Also, secondary triploids from open pollinated parental crosses flowered significantly earlier than those obtained from the hand pollinated parental crosses. Perhaps a molecular study conducted on the open pollinated secondary triploids to determine the paternity / genotype of male pollen could have provided vital information about why this is so, since the genotype of the male parents appeared to have played a vital role in determining DTF. It will be necessary also to find out in future research why the genotype of the male parent played such a critical role in *Musa.*

Author contribution

This research is the result of collaborative effort by the two authors. AT developed the concept and designed the experiment. VW conducted the field trials, collected the data, carried out the analyses and developed the manuscript.

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Competing Interests

Authors declare that there are no competing interests

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