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Full Length Research Paper

Pollen viability and germination in *Jatropha ribifolia* and *Jatropha mollissima* (*Euphorbiaceae*): Species with potential for biofuel production

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The aim of this work is to assess pollen viability using the staining technique and *in vitro* germination with different concentrations of sucrose in *Jatropha ribifolia* and *Jatropha mollissima*, contributing to the knowledge of the reproductive biology and subsidizing their conservation, management and utilization. Pollen viability was measured by dye method. Acetocarmine, acetic orcein and cotton-blue stain were used. The culture medium for pollen germination was solidificated by the addition of 1% agar combined with 0 (control), 10, 20, 30 and 40% of sucrose. The data were submitted to analysis of variance at 5% probability. All dyes used in this experiment allowed easy differentiation between fertile and non fertile pollen. The rate of formation of pollen tubes was higher in medium with 10% of sucrose for both species because the trend is that the sucrose concentration increases the supply of carbon, changes the osmotic potential and inhibits the formation of pollen tube *in vitro*.

Key words: Plant reproduction, male gametophyte, hybridization, germplasm.

INTRODUCTION

The genus *Jatropha* L. (*Euphorbiaceae*) comprises oleaginous species of high production potential (Kumar and Sharma, 2008; Achten et al., 2008; Rao et al., 2008). In spite of the strategic relevance of these plants for the Brazilian economy and agribusiness sector, there is little research on them, including *Jatropha ribifolia* (Pohl) Baill and *Jatropha mollissima* (Pohl) Baill. Both are shrubs, on average, 2 m high (Leal and Agra, 2005). It is widely used in folk medicine in Northeast Brazil, with therapeutic indications such as anti-inflammatory (Agra et al., 2008; Agra et al., 2007). Studies of pollen viability in these species are very essential for conservation, management and use.

To study different aspects of pollen development and fertility or to determine the optimal time for pollination, *in vitro* techniques need to be improved so that pollen quality can be determined reliably. The first step for successful *in vitro* manipulation of isolated microspores or pollen grains is an efficient monitoring system to determine the viability of treated grains (Vizintin and Bohanec, 2004). Two basically different approaches can be taken to estimate pollen viability: staining pollen with dyes and *in vitro* germination assay. Staining techniques aim to determine pollen enzymatic activity and membrane integrity. *In vitro* germination assays determine the actual germination ability of pollen under suitable conditions (Tuinstra and Wedel, 2000).

Pollen viability is an important factor for plant genetic variability, especially for those plants in which cross fertilization prevails over self-fertilization, like *Jatropha*, as it reveals the male reproductive capacity and enables different allele combinations (Karasawa, 2009; Divakara et al. 2010).

The appropriateness of the viability test depends on the species, since differences have been reported for optimal staining techniques (Rodriguez-riano and Dafni, 2000). Staining techniques can be used to assess the physiological condition of mature pollen and also microspores.

According to Dantas et al. (2005) and Tuinstra and Wedel (2000), assessment of pollen fertility is a

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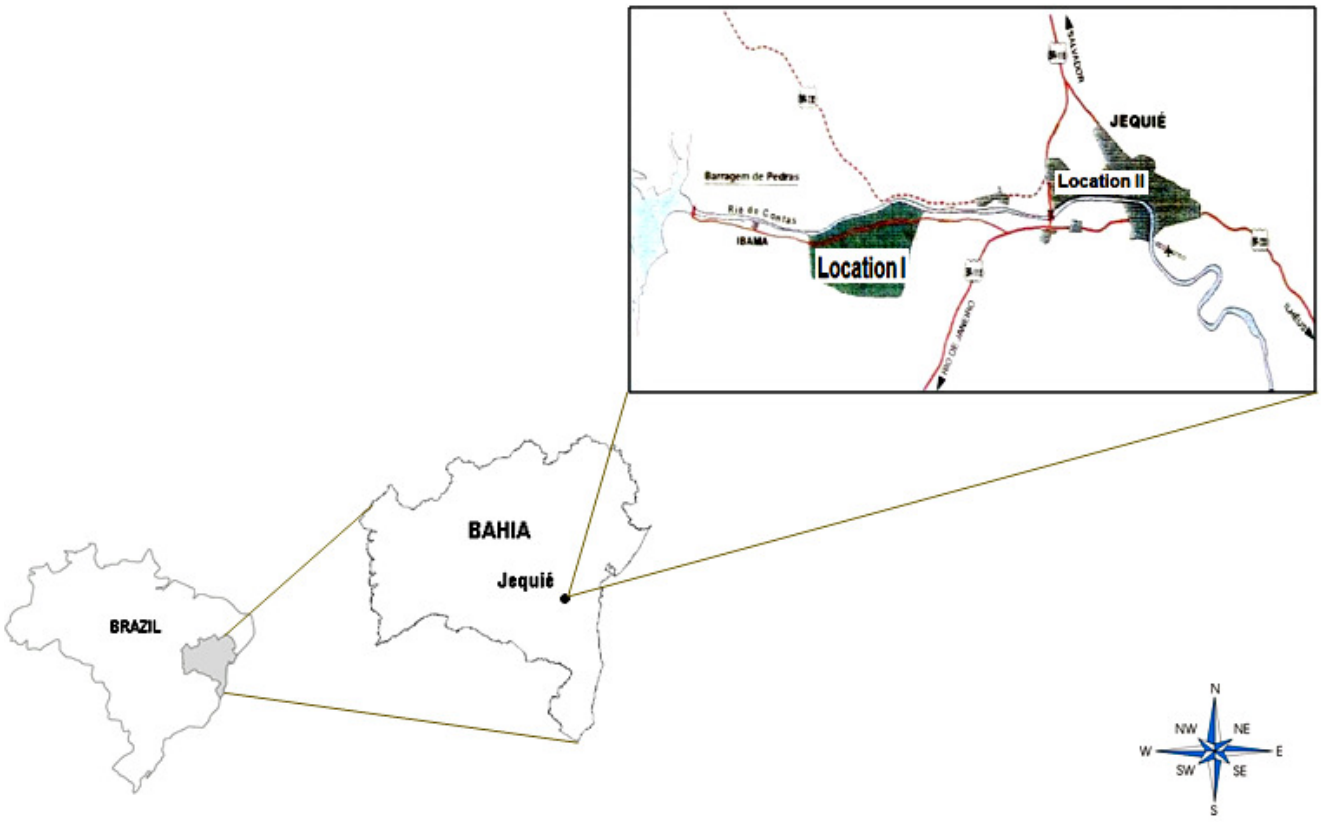


Figure 1. Study area localization in Jequié, Bahia, Northeast / Brazil, 2009.

preliminary and indispensable condition for genetic crop breeding. For these authors, information on pollen viability and germination is important for the study of reproductive biology and for the development of genetic crop programs, as it enables a greater success rate of crosses.

The *in vitro* germination is the most widely used method of testing pollen viability in breeding programs (Marcellán and Camadro, 1996). The main components of the culture medium for pollen germination have been different sugars types and concentrations (Miranda and Clement, 1990). The sugar used in the culture medium aim to provide a balance between the osmotic solution and pollen germination and provide energy to assist the process of development of pollen tubes (Loguercio, 2002).

The aim of this work is to assess pollen viability using the staining technique and *in vitro* germination with different concentrations of sucrose in *J. ribifolia* and *J. mollissima*, contributing to the knowledge of the reproductive biology and subsidizing their conservation, management and utilization.

MATERIALS AND METHODS

The study was carried out in January and December of 2009 with the species *J. ribifolia* (Pohl) Baill and *J. mollissima* (Pohl) Baill. The flowers of *J. ribifolia* and *J. mollissima* were collected in two locations, five plants in area I (84°66'32.6''S 037°64'36''W, 194m of altitude) and five plants in area II (84°65'59.8''S 038°38'82''W, 181m of altitude), making it 10 plants per specie in the municipality of Jequié, state of Bahia, Northeast, Brazil (Figure 1).

Exsiccates of the studied material were deposited in the Herbarium (HUESB) in Southwest Bahia State University (UESB), Brazil, in the numbers 4660 and 4661 for *J. ribifolia* and *J. mollissima*, respectively.

Pollen viability

At first, 20 flower buttons and 80 anthers were collected during the anthesis period (5 to 7 am) to *J. ribifolia* (Figure 2a) and (4 to 5 am) to *J. mollissima* (Figure 2b).

The floral buttons were taken to the Experimental Genetics Laboratory (LABGENEX) at the Southwest Bahia State University (UESB), Brazil. The anthers were used immediately after collection and the pollen grains were distributed with the assistance of a brush and after, were dyed with the following colorants: 1% Aceto-carmine (Nassar et al., 2000) to detect cytoplasmic content; 0.05% aniline blue (synonym: cotton blue) to detect callose in pollen walls (Hauser and Morrison, 1964) and 2% acetic orcein(Biondo and Battistin, 2001), a micronuclei stain.

Pollen grains were observed through an optical microscope (light microscope x100 magnification) and were subsequently classified as fertile or not fertile. Pollens grains that presented visibly abnormal sizes, light coloring and reduced and/or absent protoplasms

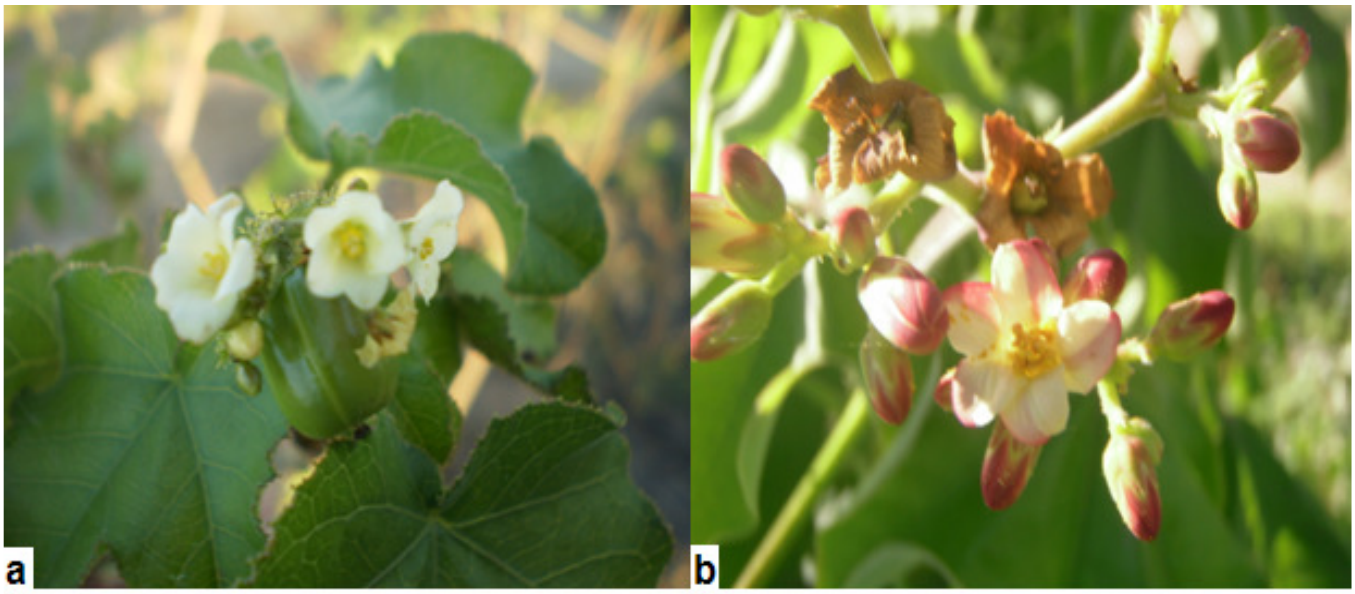


Figure 2. Male flower of (a) *J. ribifolia* and (b) *J. mollissima*, Bahia, Northeast Brazil, 2009.

were considered not fertile (it has no potential to germinate), while those that presented intact exines and strongly colored protoplasms with homogeneous distribution were classified as fertile (it has the potential to germinate).

According to Fortescue and Turner (2004), germination potential has been defined as the ability of viable pollen to germinate under suitable germination conditions. A completely randomized experimental design with 4 repetitions was used. Three hundred pollen grains were counted per repetition. The values expressing the number of viable pollen grains were transformed to arcsen $\sqrt{x(\%)\ /100}$ prior to statistical analysis. The data were subjected to analysis of variance at 5% probability using the Statistical Analysis System (SAS) statistical program 9.1.3 (2007). The experimental design for the dependent variable pollen germination *in vitro* was double factorial (species x dyes).

Pollen germination

Of the 10 plants used, 2 male flowers per plant at anthesis stage were collected, to collect pollen grains. The *in vitro* germination tests were performed in a culture medium gelled, previously autoclaved at 121°C, containing 1% agar and combined with the following sucrose concentrations: 0 (control), 10, 20, 30 and 40%. The pH was adjusted to 6.5.

The pollen of each species was distributed over the surface of the medium with a brush in order to promote a uniform distribution of the material. Soon after, the blades containing the culture medium were placed in a moist chamber simulated (petri dishes with moistened paper towels) and taken for incubation in a germination chamber B.O.D (Incubator: 411 D / FDP) in the absence of light, with controlled temperature (25 ± 1°C) for 10 h.

After this period, 100 pollen grains per blade were counted at random (experimental unit) with an optical microscope (light microscope: x100 magnification) to estimate the mean of pollen grains germinated and non germinated. For purpose of analysis, it was considered germinated, the pollen grains whose tubes reached the same or greater length than their own diameter (Báez et al., 2002).

A completely randomized experimental was used, consisting of 5 treatments and 4 repetitions. The data of *in vitro* pollen germination percentage were previously transformed to arcsen $\sqrt{x(\%)\ /100}$. The data were submitted to analysis of variance at 5% probability using the SAS statistical program 9.1.3 (2007). The experimental design for the dependent variable pollen germination *in vitro* was double factorial (species x sucrose).

RESULTS

All the dyes used in this experiment followed by the mean and standard deviation, acetocarmine (232.50 ± 26.03), acetic orcein (259.25 ± 7.50) and cotton-blue (240.75 ± 17.46) for *J. mollissima* and acetocarmine (287.25 ± 10.81) acetic orcein (200.75 ± 51.17) and cotton-blue (247.50 ± 61.12) for *J. ribifolia* showed good color to differentiate between fertile and infertile pollens. Analysis of variance of the factorial experiment indicated highly significant differences in pollen viability associated with the species and dyes (Table 1). The same analysis inferred that the staining rate of pollen grains presented a good experimental precision (CV = 9.40%).

The *in vitro* germination experiment tested different concentrations of sucrose, in which, the analysis of variance inferred that the germination rate of pollen grains presented an efficient experimental precision (CV = 23.73%). According to analysis of variance at a 5% probability level, the significant difference was between the means of the species and the interaction between species and sucrose (Table 2).

The germination was effected after three hours of incubation, and the pollen tube formation occurred only at concentrations of 0 (25 ± 3.74), 10 (29.50 ± 3.87) and 20

Table 1. Analysis of variance for variables of fertile pollen grains of species of *Jatropha* L., submitted to different dyes.

Source of variation	DF	SS	MS	F
Species	1	0.000004	0.000004	0.00
Dyes	2	0.0150	0.0075	1.42
Esp. X Dyes	2	0.0532	0.0266	5.03*
Error	18	0.0954	0.0053	
CV	9.40			

*Significant at 5% probability level.

Table 2. Analysis of variance for variables of germinates pollen grains of species of *Jatropha* L., submitted to different concentrations of sucrose.

Source of variation	DF	SS	MS	F
Species	1	0.0381	0.0381	9.11*
Sucrose	4	2.0114	0.5028	120.15**
Esp. X Suc.	4	0.0451	0.0112	2.70*
Error	30	0.1255	0.0041	
CV	23.73			

*, ** Significant at 5% and 1% probability level, respectively.

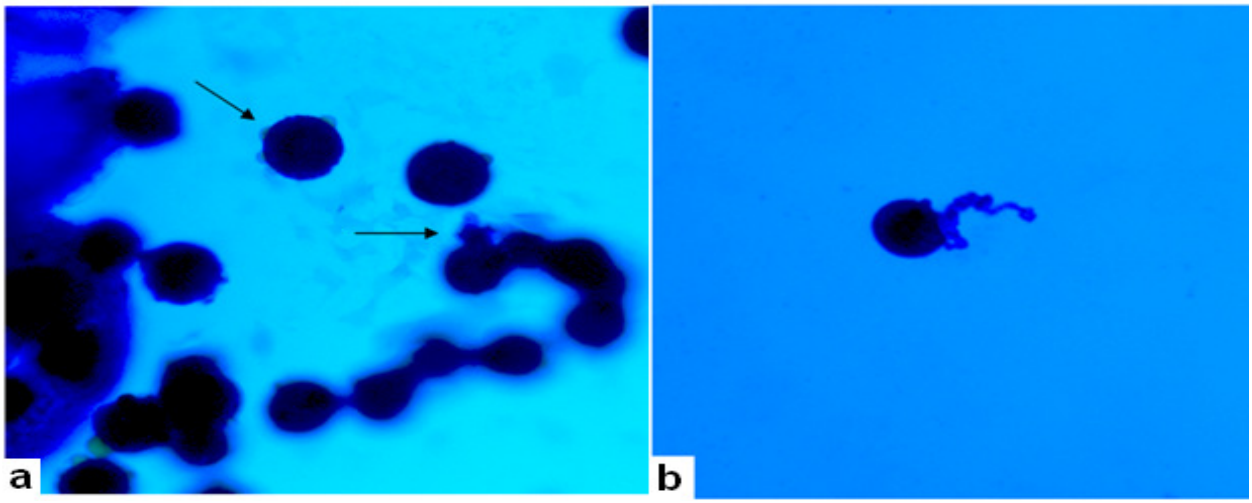


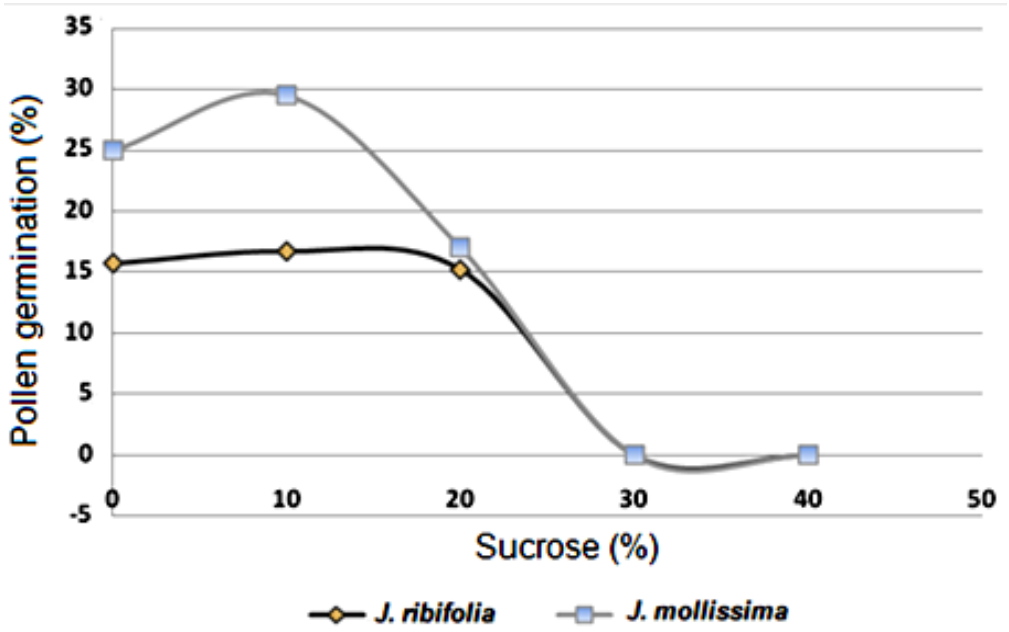
Figure 3. *In vitro* germination of pollen grains of *J. ribifolia* stained with cotton blue. (a) The up arrow showed pollen that did not germinate with exudate and down arrow showed details of the germination. (b) Germinated pollen.

(5.71 ± 17) for *J. mollissima* and 0 (15.75 ± 11.44), 10 (16.75 ± 4.57) and 20 (15.25 ± 4.64) for *J. ribifolia* (Figure 3a and b), indicating that the gametophytes have tolerance to germinate in different concentrations of sucrose, since no germination occurred at 30 and 40% of sucrose for both species. The best result was obtained in the concentration of 10% of sucrose for both species.

The polynomial regression equations of the relationships between pollen germination and sucrose concentration showed high accuracy for *J. ribifolia* (R^2 = 0.820) and *J. mollissima* (R^2 = 0.846) (Figure 4). It can be shown that the germination index tends to fall with increasing sucrose concentration.

DISCUSSION

In this work, the dye test showed a high fertility and the germination test demonstrated that a low index of pollen grains germinated for both species. Santos et al. (2006),



$Y_{J.\ ribifolia} = -1.125x^2 + 1.925x + 16.15 \quad R^2 = 0.820$

$Y_{J.\ mollissima} = -0.964x^2 - 2.164x + 31.4 \quad R^2 = 0.846$

Figure 4. Relationship between pollen germination percentage and concentration of sucrose.

Einhardt et al. (2006) and Nyine and Pillay (2007) found similar results in their experiments, emphasizing that pollen grain viability assessment through the staining method seems to express the germination potential, but not its occurrence. It may be explained by the fact that this technique overestimates the percentage of pollen tubes formed.

Though the pollen seems non-viable, the presence of some vigorous pollen tubes may indicate that it is still good enough to ensure at least a moderate efficient fruit set, despite the low germination rate (Einhardt et al., 2006). The low germination rate presented in this experiment in both species may be related, according to Nava et al. (2009) with the high temperatures during the pre-flowering/flowering, suppressing the development and inducing early degeneration of the embryo sac after anthesis.

Similar results (Techio et al., 2006) infer that the pollen, probably, almost completely lost its capacity to develop pollen tubes, it seems that only the tube germination mechanism was affected, not the fertility of the grains which was evident in this present study. In conventional plant breeding programs, simple procedures such as the one used in this experiment, were chosen for the identification of fertile pollen grains, conferring greater flexibility on scientists' work (Pio et al., 2004; Abdelgadir et al., 2009). The results obtained in this work showed that the number of pollen tubes grown in 10% of sucrose is most suitable for rapid pollen germination and formation of long pollen tubes, in addition to exercising the balance of osmotic solution and providing energy for the tube growth (Miranda and Clement, 1990; Ylstra et al., 1992).

The results of the study indicate that other means of culture may inhibit further germination of these pollen species, which for Hormaza and Herrero (1992), contribute to the selection of subsequent sporophytic generation. Furthermore, determining a way that maximizes the pollen germination is fundamental to the genetics conservation programs (Souza-Lang and Pinto Júnior, 1997).

As there was no germination of pollen in high concentrations of sucrose in this experiment, which confirms the hypothesis of Premachandra et al. (1992) where the increase in concentrations of sucrose in the culture medium increases the supply of carbon available to the culture, being the osmotic potential means changed, may inhibit, the formation of pollen tubes *in vitro*.

McCormick (2004) reported that further studies are required, like the relationship between environmental factors (temperature), DNA analyses and others mediums (boric acid and calcium nitrate). More studies with the two species used in this work will help increase the

chance of creating a hybrid of high oil content.

Studies have indicated differences in the *in vitro* pollen grain germination as a result of the complex interaction between morphology and physiology of the pollen grain and components of the medium (Gwata et al., 2003). Kakani et al. (2005) claimed that the differences observed in the germination *in vitro* and pollen tube growth of 12 cotton cultivars (*Gossypium hirsutum*) were a reflex of the variability in the cultivars. Similarly, Frazon et al. (2005) stated that there are differences between species and cultivars within the species in terms of the conditions required of a medium for *in vitro* pollen germination.

Reports that a medium containing only pure water and sucrose provided good pollen germination are rather rare. Studies by Souza-Lang and Pinto Júnior (1997) with different sugars in the pollen germination of araucaria (*Araucaria angustifolia*) showed that the highest germination percentages were observed in media without any sugar. Probably, the concentrations used may have had no effect, owing to the osmotic imbalance between the pollen and the culture medium.

For Adhikari and Campbell (1998), pollen viability is strongly influenced by temperature, moisture, genotypic differences, plant vigor and physiological stage, and flower age. Here, the different responses of pollen viability can be ascribed to the genotype, since temperature and moisture conditions were controlled and the male inflorescences were sampled at the same physiological stage.

Data on the viability and development of pollen grains are fundamental for studies of reproductive biology and genetic breeding of *Jatropha*, ensuring safer crosses performed to generate new hybrids and/ or increase the fertility.

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