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Pollen fertility in wild × cultivated F_1 hybrid of cowpea (*Vigna unguiculata*)

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Abstract

Hybridization is progressively more recognized as an important process in the evolution of plant populations and species. In order to produce F_1 hybrid plants, three subspecies of wild cowpea namely *ssp. spontanea, ssp. stenophylla* and *ssp. tenius* were crossed with the cultivated cowpea subspecies namely *ssp. unguiculata* (524B). The resulting F_1 hybrids were evaluated for pollen fertility, determined from more than 2000 pollen grains. Pollen fecundity in hybrid groups (74.45 ± 1.54) was significantly lower ($t = 9.987, df = 51; p < 0.0001$) compared to those of parent groups (95.27 ± 0.29). Between parents groups, one-way analysis of variance test at $p < 0.050$ showed no significant difference in Pollen fertility ($F = 2.450; p = 0.093$) while significant difference in pollen fecundity was found between hybrid groups ($F = 8.063; p < 0.010$). *spontanea* (♀) × (♂) 524B F_1 hybrid produced significantly more fertile pollens compared to *tenius* (♀) × (♂) 524B and *stenophylla* (♀) × (♂) 524B F_1 hybrids. This indicates that with the cultivated cowpea (524B), reproductive barrier are significantly more pronounced with wild forms *ssp. stenophyllla* and *ssp. tenius* compared to *ssp. spontanea*. *ssp. spontanea* is therefore the wild cowpea subspecies more closed to the cultivated cowpea (*ssp. unguiculata*) compared to *ssp. tenius* and *ssp. stenohylla*. Implications of these results in the light of the development of a CMS (Cytoplasmic Male Sterile) plant in hybrid cowpea seed technology are discussed.

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Introduction

Cowpea (*Vigna unguiculata*) is an essential grain legume in tropical and subtropical regions. It provides inexpensive proteins, feeding people and their livestock and the next crop (Singh *et al.*, 1997). It therefore contributes significantly to food security in the African continent. Cytological studies reveals that Cowpea is a diploid plant species (2n = 22) (Pasquet, 1999). This legume according to Pasquet (1999) is composed of cultivated cowpea (*ssp. unguiculata)* and 11 wild / weedy forms named *ssp. spontanea*, *ssp. alba, ssp. pubescens, ssp. stenophylla, ssp. tenius, ssp. dekindtiana*, *ssp. burundiensis, ssp. letouzeyi, ssp. pawekiae, ssp. aduensis* and *ssp. baloulensis*. The wild / weedy forms of cowpea are only encountered in the African continent (Pasquet, 1999). Cultivated plants and wild relatives represent an interesting system from agricultural and evolutionary points of view. Wild species of crop plants are placed in different gene pool based on their cross ability with the cultivated species (Aliyu, 2005). Wild plants that hybridize easily with the cultivated plant belong to the same gene pool with the cultivated crop. When hybridization is difficult with the cultivated plant, both species should belong to different gene pools (Aliyu, 2005).

The important role of hybridization on the origin of plant populations is well documented (Hugues *et al.,* 2007).Gene flow and natural hybridization between plant species are established to be of significant consequence in the evolution and ecology of plant species (Lewis, 1966). It can also result to the evolvement of new plant species (Rieseberg *et al.,* 2003). With the concern of biosafety for genetically modified crops, there is progressively more and more interest in crop x wild and wild x crop hybridization. This is simply because gene flow can be an avenue for transgene escape (Ellstrand and Holfman, 1990). To understand better the dynamics of hybridization and introgression within *Vigna unguiculata*, we hybridized three wild subspecies with the cultivated cowpea and estimated one main component of fitness of the offspring that is the pollen fertility.

Several staining techniques have been used to estimate pollen fertility in plant species. Aceto-carmine stain (Pearson and Harney, 1984; Mercardo *et al.,* 1997; Qureshi *et al.,* 2009), Potassium-Iodine stain (Waheed *et al.,* 2013), Aniline blue stain (Springer *et al.,* 1989; Leppala and Savolainen, 2011) and Alexander's stain (Bures *et al.,* 2010; de Paula *et al.,* 2014) are the most widely used staining techniques for pollen fertility estimation. The central principle of these reactions is due to the affinity of some acid in plant cells with basic dyes, coloring pollen (Asghari, 2000). Data on fertility of pollen is an important means to differentiate the potential hybrid and parental plant. The level of hybrid fertility might provide a few signals of the extent of heritable connection among its parents. Therefore, data on pollen fertility for any plant species is essential for plant breeders. The present study was undertaken to determine pollen fertility status using in vitro pollen viability tests by Alexander staining technique (Alexander, 1969) in twenty-four accessions of wild and cultivated cowpea from different gene pools and thirty-four wild (♀) × (♂) cultivated F_1 Hybrid of *Vigna unguiculata*.

Material and methods

Plant material

The experimental plant materials are parents comprising wild seeds accessions and 6 seeds of a breeding line of *ssp. unguiculata* from the University of California Davis named 524B. The wild seeds include *ssp. spontanea* (SP46, SP181, SP215, SP219, SP 278 and SP263); *ssp stenophylla* (SP340, TVNU458, TVNU814, TVNU1148, TVNU1448 and TVNU1468) and *ssp. tenius* (MT5, MT29, MT38, MT206, SP73 and SP161).

Hybridization: Experimental crosses

Hybridization block was set for the above plant material in the screen house at the Muhaka field station of the International Centre of Insect Physiology and Ecology located at 4°19.5'S 39°31.5'E, 32 km south-southwest of Mombasa in Kenya. Hybridization was carried out at flowering stage. Flowers from the female parent were emasculated prior to pollen transfer. Pollen grains from dehiscent anthers from designated male parent were applied on the stigmatic surfaces of the already emasculated flowers. The newly hand pollinated flower was then bagged with flowers sepals of the male plant flower to prevent the arrival of any foreign pollen. The F_1 Hybrid seeds were harvested at maturity.Seeds from 34 wild (♀) × (♂) cultivated F_1 hybrids were produced.

Cultivation of F_1 plants and related parents

Parents as well as F_1 seeds were pre-germinated in imbibed Wattman Paper for 3 to 5 days and transferred in the screen house for development. Plants were provided with 5 g of N-P-K (16-16-16) as proposed by Kareem *et al.* (2013) Plants were watered regularly till flowering and maturity

Staining and pollen fertility assessment

At flowering, open flowers with dehiscent anthers were collected in the morning between 08 and 09 AM. Flowers from each hybrid and parents were harvested and brought to the laboratory. Pollen grains from dehiscent anthers were placed in lame slide and one drop, approximately 50 µl of Alexander solution (Alexander, 1969) was added. A clean cover clip was gently lowered on stained pollen grains in such a way as to avoid splashing and air trapping. For each parent and hybrid, flowers were collected from at least three different plants. For each plant, three slides were prepared. For each slide ten randomly selected fields were observed and pollens were counted under the light microscope at 100 X magnification. A total of > 2000 pollen grains were counted on each slide and classified as fertile or sterile according to their staining behavior. All Reddish and dark stained pollens were scored as fertile while green and irregularly shaped unstained pollen grains were sterile, characteristics that were also used by Falusi *et al.* (2001) when studying sesamum species, Akaffou *et al.* (2014) with the study of pollen viability in Coffea or in Asteraceae species (Qureshi *et al.,* 2009). Pollen fertility was calculated by dividing the number of fertile pollen grains by the total number of grains counted.

Statistical analysis

Pollen fertility was determined for the parents and the hybrid plants. Data were statistically analyzed using Prism, version 6.01 of MacOS. Student's t-test and one-way analysis of Variance (ANOVA) followed by Tukey test were applied. Statistical analysis of pollen fertility was carried out for each plant group. Probability levels were considered to be statistically significant (*) at $P \leq 0.05$, and highly significant (**) at $P \leq 0.01$. Differences were not considered to be statistically significant at $P > 0.05$.

Results

Pollen fertility in Parents plants

Pollen fertility in cultivated cowpea *ssp. unguiculata* (524B) ranges from 93.3 to 96.4% with an average of 95.11% (Table 1). In wild cowpea, pollen fertility was 96.18% (ranges 95.12 – 89.74) with *ssp. spontanea* ; 94.19 % (ranges 92.8 – 96.1) with *ssp. tenius* and 95.61% (ranges 93.1 – 97.7) with *ssp. stenophylla*(Table 1). Analysis of variance (ANOVA) did not show any significant difference between parent groups (F 2.45, P > 0.050; table 2). Comparing wilds and cultivated parents, Student t-test did not revealed any significant difference (P > 0.050, data not shown).

Table 1. Pollen viability estimates in Cultivated (524B), wilds and Wild (♀) × (♂) Cultivated *Vigna unguiculata.* *Indicates significant difference in pollen fertility between parents and F_1 hybrid.

		N	Minimum	Maximum	Mean ± SE
Parents					
	ssp. unguiculata (524B)	6.00	93.30	96.40	95.11 ± 0.42
	ssp. Spontanea	6.00	95.12	98.74	96.18 ± 0.53
	Ssp. Tenius	6.00	92.80	96.10	94.19 ± 0.51
	ssp. Stenophylla	6.00	93.10	97.70	95.61 ± 0.66

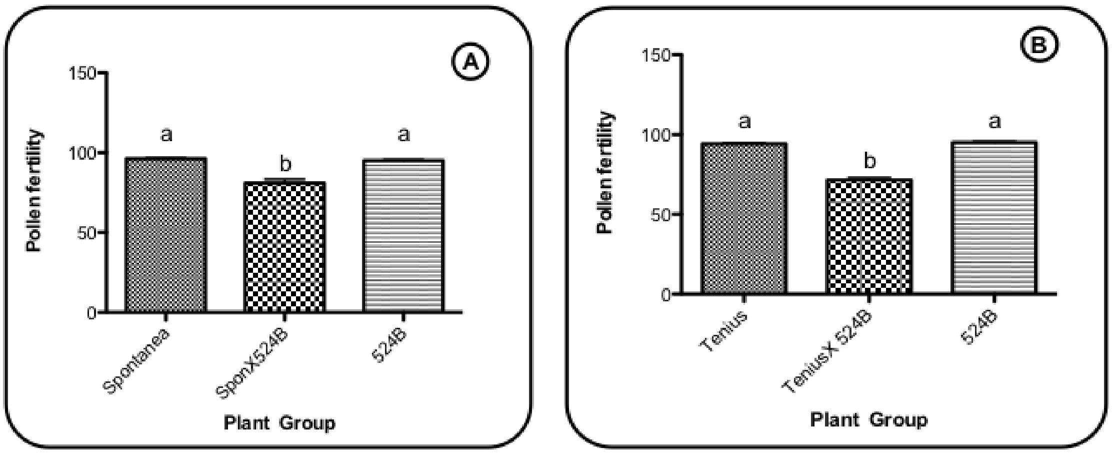
		N	Minimum	Maximum	Mean ± SE
	Parents	**24.00**	**92.80**	**98.74**	**95.27 ± 0.29***
F1 Hybrid	*ssp. spontanea X ssp. unguiculata (524B)*	13.00	71.01	98.36	80.91 ± 2.42
	ssp. Tenius X ssp. unguiculata (524B)	13.00	58.60	78.40	71.48 ± 1.39
	ssp. stenophylla X ssp. Unguiculata (524B)	8.00	56.70	81.24	68.78 ± 3.11
	F1 Hybrids	**34.00**	**56.70**	**98.36**	**74.45 ± 1.54***

Table 2. Results of ANOVA test to identify effects of parental groups and F1 Hybrid combination on pollen fertility in *Vigna unguiculata*. **: Significant at P = 0.01 probability level. NS: Not significant at P = 0.05 probability level.

Dependent variable	Factor		df	SS	MS	R^2	F	P
	Parent Groups	Between Parents Groups	3	12.87	4.829	0.269	2.45	0.093^{NS}
		Within Parent Groups	20	35.02	1.751			
Pollen fertility	F1 Hybrid Groups	Between Hybrid Groups	2	915	457.5	0.342	8.063	0.0015^{**}
		Within Hybrid Groups	31	1759	56.74			

Pollen fertility in wild × cultivated F_1 hybrid plants

With *ssp. spontanea* (♀) × (♂) *ssp. unguiculata* (524B) F1 hybrid, pollen fertility ranges from 71.01 to 98.36 %, mean at 80.91 %. Pollen fertility for *ssp. tenius* (♀) × (♂) *ssp. unguiculata* (524B) F1 hybrid ranges from 58.60 to 78.40% with mean at 71.48 %. Pollen fertility for *ssp. stenophylla* (♀) × (♂) *ssp. unguiculata* (524B) F1 hybrid was 68.78 % and ranging from 56.70 to 81.24 %. Using student's t-test, pollen fertility in parent plants (95.27± 0.29 %) was significantly higher ($t = 9.987$, $df = 51$, $P < 0.01$) compared to those of F1 hybrid plants (74.45 ± 1.54 %). One-way analysis of variance revealed significant difference in pollen fertility between wild (♀) × (♂) cultivated F1 hybrid plant groups ($F = 8.063$, $P < 0.01$, Table 2). Using Tukey test, each F1 hybrid plant were having significantly low pollen fertility comparing to parents (Fig. 1A, 1B and 1C). *ssp. spontanea* was significantly more closed to the cultivated cowpea (*ssp. unguiculata*) compared to *ssp tenius* and *ssp. stenophylla* (Fig. 1D)



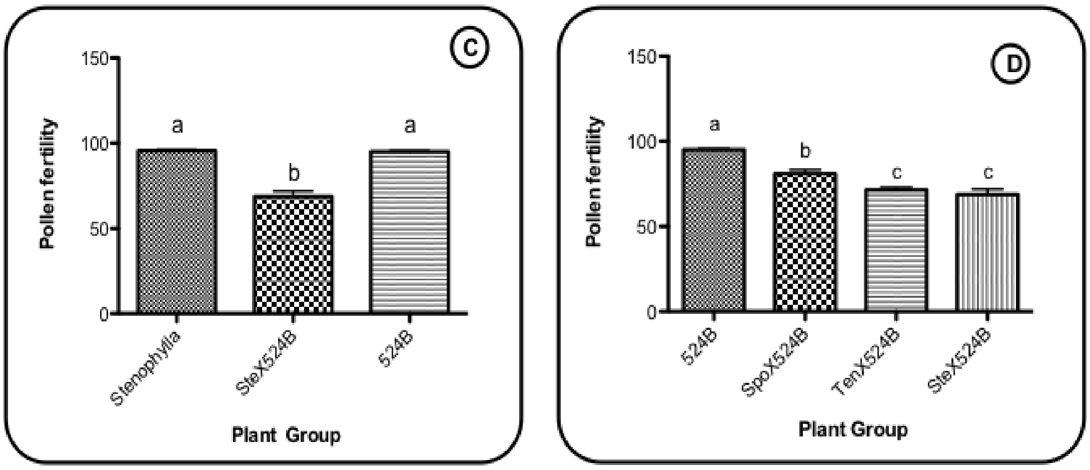


Fig. 1. Pollen fertility in cultivated, wild cowpea and F1 hybrids. Different letters indicate significant differences between groups (Tukey test, P < 0.05). Same later indicate no significant differences between groups, and error bars represent standard error.

Discussion

Creating genetically modified crop and new plant varieties have been the concern of many plant breeders all over the world (Prakash, 2001; Key *et al.,* 2008). New plant varieties generally arise through gene transfer and hybridization (Ting *et al.*, 2014). The success of hybridization includes the ability of the donor plant to produce viable pollens and the duration time of the pollen viability (Ting *et al.,* 2014). Pollen fertility as well as allele frequencies and genetic diversity indices can greatly vary between plant species as they can also depend on environmental agents, such as temperature, rainfall distribution and relative humidity (Yang *et al.,* 2004; Kouam *et al.,* 2012)

Pollen fertility is known to be one of the fitness components in many hybrid plant species (Stebbins, 1958). In this study, there is a reduction of pollen fertility in F_1 hybrid compared to parents. Pollen of F_1 hybrid between wild and cultivated cowpea is only 74.45 ± 1.54 % fertile whereas pollen of the parents are 95.11 and 95.33 % fertile, respectively for the cultivated and wild parent. This tendency of reduction of fertility in F_1 hybrid plants was also reported in Brassica plants (Hauser *et al.,* 1998); in rice (Abebrese *et al.,* 2011; Song *et al.,* 2004); in solanum species (Oyelana and Ugborogho, 2008); in arabidopsis lyrata subspecies (Leppala and Savolainen, 2011); in Helianthus species (Terzic *et al.,* 2006) and in sesamum species (Falusi *et al.,* 2001)

Knowledge of the genetic basis of hybrid compatibility or incompatibilities is important in order to understand the evolutionary dynamics and the process of species divergence (Sweigart *et al.,* 2006). The reduction of pollen fertility in the F_1 plant compared to parent plants implies that there are some little difficulties in crossing between cowpea subspecies. However, pollen fertility in Hybrid remains high, at about 75%. This shows that hybridization success is highly possible in nature within cowpea subspecies as reported by Kouadjo *et al.* (2007) and Lelou *et al.* (2011). The genetic disparity between crossing species is expected to contribute to the fitness or success of hybrids (Burgess and Husband, 2004). Significant differences between mean percent F_1 Hybrid pollen fertility from wild (♀) × (♂) cultivated cross indicates that the different wild *vigna unguiculata* used for this study belong to different gene pool.Data on pollen fertility in Wild (♀) × (♂) Cultivated F_1 hybrid from different cowpea genotypes showed that *ssp. spontanea* (♀) × (♂) 524B F_1 hybrid had a high pollen fertility (80.91%), significantly different from the pollen fertility of *ssp. tenius* (♀) × (♂) 524B F_1 hybrid (71.48%) and *ssp. stenophylla* (♀) × (♂) 524B F_1 hybrid (68.78%). These results suggest a weak incompatibility barrier and a high genetic affinity between the cultivated cowpea and *ssp. spontanea*. Several reports and studies (Pasquet, 1999, 2000; Kouadjo *et al.,* 2007; Coulibally *et al.,* 2002; Ba *et al.,* 2004) also revealed a high genetic resemblance between *ssp. spontanea* and the cultivated cowpea (*ssp. unguiculata*). Hybridization between *ssp. tenius* and the cultivated form *ssp. unguiculata* generated a F_1 hybrid with significant reduction in pollen fertility. This suggests the existence of a more important genetic divergence. The lack of significant difference between pollen fertility of *ssp. tenius* (♀) × (♂) 524B F_1 hybrid (71.48%) and *ssp. stenophylla* (♀) × (♂) 524B F_1 hybrid (68.78%) indicates that *ssp. tenius* and *ssp. stenophylla* belong to the same gene pool. This corroborate with the findings of Pasquet (1999) on the organization of *Vigna unguiculata*and ranging *ssp. tenius* and *ssp. stenophylla* in the same gene pool and *ssp. spontanea* more close to the cultivated cowpea *ssp. unguicultata*.

Conclusion

This study shows the reduction of pollen fertility in F_1 hybrids compared to parent plants and significant difference in pollen fertility between different F_1 hybrid plants. These results contribute to the increasing knowledge of the reproductive isolation in *Vigna unguiculata*. To further understand the generality of these results, additional crosses and pollen fertility tests between several different subspecies will be conducted with the aims of setting a base line for the development of CMS (Cytoplasmic Male Sterile) plant to be use in cowpea hybrid seed technology.

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