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Polyploidy can confer superiority to West African *Acacia senegal* (L.) Willd. trees

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Abstract

Polyploidy is a common phenomenon in the evolution of angiosperms. It has been suggested that polyploids manage harsh environments better than their diploid relatives but empirical data supporting this hypothesis are scarce, especially for trees. Using microsatellite markers and flow cytometry, we examine the frequency of polyploids and diploids in a progeny trial testing four different populations of *Acacia senegal*, a species native to sub-Saharan regions of Africa. We compare growth between cytotypes and test whether polyploid seedlings grow better than diploids. Our results show that polyploids coexist with diploids in highly variable proportions among populations in Senegal. *Acacia senegal* genotypes were predominantly diploid and tetraploid, but triploid, pentaploid, hexaploid and octaploid forms were also found. We find that polyploids show faster growth than diploids under our test conditions: in an 18 years old field trial, polyploid superiority was estimated to be 17% in trunk diameter and 9% in height while in a growth chamber experiment, polyploids grew 28 % taller, but only after being exposed to drought stress. The results suggest that polyploid *A. senegal* can have an adaptive advantage in some regions of Africa.

Key words: adaptation - arid zone trees - drought stress – flow cytometry - microsatellite markers - morphological differentiation - *Senegalia senegal*

1. Introduction

Polyploidy, the achievement of more than two sets of chromosomes through gametic non-reduction and to a lesser degree somatic doubling has important ecological and evolutionary consequences for speciation (Madlung, 2013). In nature, polyploidy arises via intraspecific genome doubling (autopolyploidy) or merging of genomes of distinct species through hybridization and chromosome doubling (allopolyploidy) (Stebbins, 1950). Most angiosperms are believed to have undergone one or more polyploidization events (Soltis *et al*., 1999). It has been estimated that polyploids form at the frequency of approximately 1 per 100,000 individuals (Ramsey & Schemske, 1998; Levin, 2002) and that 2 – 4 % of all speciation events involve polyploidization (Otto & Whitton, 2000). The high level of polyploidization in the evolutionary history of flowering plants suggests that polyploidy plays an important role in adaptive evolution of plants in natural populations (Van de Peer *et al*., 2009). Successful polyploidization is generally accompanied by morphological, phenological, physiological, and ecological changes in plants (Levin, 2002), and may produce individuals that can tolerate fluctuating environments (Soltis *et al.*, 2004; Prentis *et al*., 2008), make use of new niches or by other means become more successful than their progenitor species (Leitch & Leitch, 2008).

In theory, local co-occurrence of intraspecific cytotypes is evolutionary unstable, driving the minority cytotype towards extinction unless cytotypes have different ecological preferences or strong prezygotic barriers are present between ploidy levels (Husband 2000, Kennedy *et al.*, 2006). Nevertheless, co-distribution of individuals belonging to different ploidy levels in heteroploid species (species with different levels of ploidy) is not uncommon and has been reported in *e.g. Solidago altissima*, *Ranunculus parnassifolius* and *Centaurea phrygia, C. stoebe* (Halverson *et al.*, 2008; Cires *et al*., 2010; Koutecky *et al*., 2012). With the recent introduction of flow cytometry is it now possible to explore the cytotype dynamics in species with mixed ploidy levels in terms of cytotype distribution, hybridization and segregation.

Morphological changes in polyploids include a general increase of cell size with increased levels of ploidy (e.g. Kudo & Kimura 2002), sometimes leading to changes in the dimensions of plants, such as larger leaf, flower and fruit sizes compared to diploids (Maherali *et al.* 2009, Pettigrew *et al.* 2012). Also micro-morphological changes occur, including larger but more dispersed stomata in polyploids than in diploids (Mishra 1997, Pettigrew *et al.* 2012). Such changes are likely to affect plant environment interactions, for example through modification of gas exchange. It was earlier suggested that polyploids withstand harsh environments like subarctic regions, high elevations and xeric environments better than diploids (Love & Love, 1949) perhaps due to their higher levels of heterozygosity and genetic diversity (Lowry & Lester, 2006). This was supported for example by observations of higher colonization potentials in polyploids, increased frequencies of polyploids from warmer to colder latitudes (Manton, 1934, Hagerup, 1939, Brochmann *et al*., 2004, Lowry & Lester, 2006) and larger ecological amplitude of polyploids compared to diploids (e.g. Liu *et al*., 2011, Schlaepfer *et al*., 2010).

Recent studies have found that polyploid cytotypes in heteroploid species complexes of herbaceous plants have better drought tolerance than their diploid progenitors (*Chamerion angustifolium*, Maherali *et al*., 2009; *Brachypodium distachyon*, Manzaneda *et al.*, 2012), although Buggs & Panell (2007) found that diploid *Mercurialis annua* performed better than hexaploids across a range of natural sites and that the cytotypes did not differ in performance under drought stress. Increased drought tolerance may be related to higher resistance towards cavitation in the xylem as discovered in *Atriplex canescens*, a shrub species from the deserts of Southwestern U.S. (Hao *et al*., 2013). Nevertheless, it is still discussed under which circumstances polyploidy confer higher fitness (Madlung, 2013), and a comparison of many North American diploid and polyploid species showed no significant differences in extent of range or geographical distribution between ploidy levels

(Martin & Husband, 2009). Unfortunately very few studies showed the relative performance of diploids and polyploids under controlled conditions (Soltis *et al.* 2010).

Variation in ploidy level within species is also known from trees (e.g. the *Adansonia digitata*/*A. kilima* complex, Pettigrew *et al.*, 2012, *Betula papyrifera*, Li *et al.*, 1996, *Populus tremula*, Johnsson 1940, *Acacia mearnsii*, Beck *et al.* 2003). Because increased cell size is likely to influence hydraulic properties via the influence on conduits (see e.g. Hao *et al*., 2013, Maherali *et al*., 2009), studies on trees with their massive xylem, large size and long potential exposure to climatic extremes are particularly interesting. Yet there are few studies comparing performance of trees with different ploidy levels, and the studies that exist focus on short-term responses of seedlings to stress (Li *et al.*, 1996, Li *et al.*, 2009). Studies of performance of mature polyploid versus diploid trees are to our knowledge absent.

Recently it was discovered that *Acacia senegal* (L.) Willd. exists in different levels of ploidy. The species grows naturally in the semi-arid sub-Saharan regions of Africa as well as in India and Pakistan and plays an important role in agroforestry systems by providing fuel, fodder for livestock and restoring soil fertility besides producing Gum Arabic. Gum Arabic is a natural exudate collected from branches and stems after tapping during the dry season, and is only produced when the species is grown under dry conditions (Wekesa *et al*., 2009). The gum provides an important income for rural people. Most of the populations across the distribution area seem to be composed of diploid trees, but tetraploid individuals (2n=4x=52) were discovered in populations from Mali, Sudan and Ethiopia (Assoumane *et al*., 2013). Due to a limited sample size within each population the ratio between diploid and polyploid individuals was not resolved. Based on chloroplast data, the authors suggest that polyploid *A. senegal* is allopolyploid (Assoumane *et al*., 2013). The parent species that hybridized with diploid *A. senegal* may have been *Acacia laeta* reported to be a triploid hybrid (3x = 39) between *A. senegal* and *A. mellifera* (Ross, 1979), but the origin and type of polyploidy in *A. senegal* is still not verified.

Investigating a progeny trial with mature *A. senegal*, we discovered that the trees had mixed ploidy levels (Diallo *et al*., 2015). As the trial was established with the purpose of breeding for increased gum production, the trees were planted in an experimental design and thus represent a unique possibility for studying the long-term performance of di- and polyploid trees. In this paper, we specifically 1) explore the distribution of cytotypes in trees originating from four different locations, and in their corresponding offspring, 2) compare the growth performance of trees with different ploidy levels, 3) compare the growth of seedlings with different ploidy level under drought stress and 4) compare the morphology of plants with different ploidy levels.

2. Materials and methods

2.1. Study species

Acacia senegal (L.) Willd. is a multipurpose tree that belongs to the family Fabaceae. Recently, it has been suggested to transfer the species to the new genus *Senegalia* (Maslin, 2006). Here we maintain the rule of first priority and thus the name *Acacia senegal*. The species is described as consisting of the four varieties *senegal*, *kerensis*, *rostrata* and *leiorhachis* based on differences in inflorescence axis, pod and tree shape and phenology (Odee *et al*., 2012). Only *A. senegal* var. *senegal* has been reported in Senegal.

2.2. The field trial

The plant material of *A. senegal* used in this study originated from a progeny trial in Senegal established in 1994 in Dahra, Senegal (15˚ 20' N and 15˚ 28' W). The annual precipitation at the site is approximately 410 mm, and the annual mean temperature is 27°C (climate data estimated from Worldclim based on the 1950-2000 period, see Hijmans *et al*. 2005). The soil at the site is sandy, and the natural vegetation in the area consists of mainly *Acacia tortilis* and *Balanites aegyptiaca* (Pontanier *et al*. 2003).

In November and December 1993, seeds were collected from four populations (provenances) representing the natural distribution area of the species in Senegal (Fig. 1): Ngane, located in the centre of Senegal and characterized by saline soils and 620 mm of rainfall; Diamenar, located in a dryer region in the north with 300 mm of rainfall; Daiba and Kidira, located in the north-eastern and south-eastern parts with 430 mm and 600 mm of rainfall, respectively. At each site seeds were collected from 15 trees considered to have desirable phenotypes based on superior health, trunk diameter, crown diameter and height. In the natural stands diploids and polyploids are indistinguishable with the naked eye, implying no bias for or against any type during seed collections. Seeds were kept in separate lots for each mother tree, thus giving 60 seedlots, and in 1994 seeds were pretreated in sulfuric acid (98 %) for 6 min and sown in polyethylene bags with nursery soil. Two seeds were sown per bag, and in cases where both seeds germinated, one of the seedlings was randomly selected and removed. Seedlings were raised in a nursery and in August 1994 (during the rainy season), 30 healthy seedlings per seedlot were selected and planted at the Dahra site. Prior to plantation, weeding and clearing were undertaken in the site. The trial was established in a randomized complete single tree block design with all seedlots represented by one tree in each block, replicated thirty times (30 blocks). The initial number of plants was thus $4 \times 15 \times 30 = 1800$ trees. Trees were spaced 5 x 5 meters from each other.

One year after planting, in August 1995, survival and height of all trees were assessed. Height was measured as vertical height from the ground to the top of the tree. In February 2012, a second assessment was conducted on the 634 surviving trees. The maximum vertical height was determined using a height rod, trunk diameter at 30 cm from the soil surface was assessed using a diameter tape, and crown diameter was estimated as the average of two perpendicular measures of the edge of the crown projected to the ground. In 2012, cambium samples were taken from every tree for assessment of the ploidy level (see later).

2.3 Growth chamber experiment

In November 2012, 108 pods were collected from 76 parent trees in the field trial. The parent trees were selected to cover all four sites of origin and – to the extent possible – different levels of ploidy in all sites of origin. One pod was randomly chosen from each tree except for trees from Ngane where 1-6 pods were collected per tree (due to higher frequency of polyploidy in this provenance as described below).

The number of seeds per pod was counted and seed dimensions (length and width) were measured using a Vernier caliper. Seeds were pretreated with sulfuric acid (95-97 %) for 10 min to release seed dormancy, kept under sterile conditions in a laminar hood (Thermo Scientific, SAFE 2020, Germany) and germinated in boxes containing sterilized vermiculite and incubated at 29 ºC and 16 h photoperiod in a growth chamber. The germination rate was registered, and the ploidy level of all seedlings was determined (see below).

To compare the growth between different levels of ploidy, 132 seedlings were transplanted in peat soil (Plugg och Såjord) from Weibulls Horto AB, Sweden. 500 g of soil was filled in 17 plastic boxes each. The seedlings consisted of 83 diploids, 46 tetraploids and three hexaploids. Each box was

considered as one block and contained eight seedlings representing different sites of origin (provenance), descendance and ploidy levels. By descendance we here understand all seedlings descending from a single mother tree in the Dahra field trial. Because of the restricted number of polyploid seedlings in some sites of origin (provenances), the design was imbalanced, but each box contained at least three sites of origin and one set of diploids and polyploids from the same site of origin. For example, block no. 1 included four tetraploids and one diploid from the Ngane origin, and one diploid of each of the Daiba, Diamenar and Kidira origins, while block no. 17 contained one diploid and one tetraploid of the Kidira origin, and three diploids of each of the Daiba and Diamenar origins. Despite the imbalance in the trial, this design allowed us to assess the growth of the different levels of ploidy because there was always at least one pair of dipoid and polyploid seedlings from the same origin in each block. We planted seedlings in boxes to make sure that all plants in a box were exposed to the same water level irrespective of plant size, leaf area and stomatal conductance (Verslues et al. 2006).

The initial weight of seedlings was recorded before transplanting into soil. We first compared the growth under well-watered conditions (85% of field capacity) for five weeks in the growth chamber at 16 h photoperiod. The temperature varied between 28 and 33 ºC, while relative air humidity ranged between 47 and 71%. Boxes were weighed every day and the amount of water lost by evapotranspiration was added to maintain 85% of field capacity. Plant height was measured weekly from the soil surface to the apical bud, and the numbers of leaves and branches were counted after three weeks. Leaf length, leaflet length and width were measured after five weeks of growth under well-watered conditions. Likewise, stomatal size and density were determined on 50 plants (diploid and polyploid) and two randomly chosen leaflets per seedling. Leaflets were stained with Toluidine Blue O dissolved in 0.05 % of benzoate buffer and water at pH 4.4 (O'Brien & McCully, 1981) and viewed with a microscope (Olympus Cx40 RF 200, Japan) under x 40 magnification. Two microscopic grids were examined per leaflet, totaling 200 counts. To determine the size of stomata, 20 random individual stomata per seedling were measured (scale bar 100 µm) at magnification x 40 and the mean stomatal length and density was calculated.

After five weeks, drought stress was applied by reducing the amount of added water to 47% of field capacity. In a pilot study, this was shown to be close to the wilting point. Again, in order to maintain the field capacity at 47%, boxes were weighed every day and the amount of water lost was added. Plant height was recorded weekly for six weeks and the fresh and dry biomasses of seedlings (after drying at 80ºC for 48 h) were assessed at the end of the experiment.

2.4. Ploidy level assessment

For 59 trees from the Dahra field trial, twigs of 20 cm length with vegetative buds were collected in April 2014 and placed in a lab with their proximal end in water. After two weeks of forcing, one complete leaf was collected from each twig and analyzed by flow cytometry. Seeds descending from the mother trees in the Dahra field trial were germinated as described above, and flow cytometry was performed on 3 weeks old leaves in the lab.

Flow cytometry was performed using a Partec PA II flow cytometer equipped with an HBO-100 mercury arc lamp (Partec GmbH, Germany) and filter combination for DAPI staining (Partec 06-03-310). Fresh leaf samples of two-weeks-old plants or from forced twigs were chopped for 30 s in a petri dish containing 0.6 ml of Citric acid buffer and left for 5 min to allow nuclei release. The nuclei were stained by adding 2.5 ml of fluorescent solution containing 5 µM DAPI (4.6-Diamino-2-phenylindol dihydrochlorid) and left for another 5 min (Otto, 1990). The suspension of nuclei was

passed through a nylon filter with pore size 50 µm to remove large debris. The DAPI binds to the A-T bases of DNA and the intensity of the fluorescence emitted will reflect the number of bounds and therefore also the DNA content in such DAPI-labelled nuclei. The relative fluorescence of total DNA of single nuclei was analyzed and in each sample the DNA content of 5000 nuclei was checked. Samples of *Miscanthus sinensis* with known ploidy (diploid) was used as an internal standard. The standard produced two peaks: a major peak corresponding to 2x DNA quantities of the majority of the cells and a minor peak corresponding to 4x DNA quantities from cells in mitotic interphase.

The gain was adjusted so that the peak of diploid *A. senegal* was localized on channel 50 corresponding to one large (2C) and one small (4C) peak. Plants were regarded as tetraploids if histograms showed one major 4C peak, a small 8C peak and no 2C peak.

To assess the exact DNA content of the genome, a subset of 26 leaf samples from 13 DAPI-examined plants (3 diploids, 3 triploids, 3 tetraploids, 1 pentaploid and 3 hexaploids) was analyzed using flow cytometry with propidium iodide dye according to the protocol by Doležel *et al.* (2007).

All 634 living trees in the field trial were genotyped with 8 polymorphic microsatellite markers (SSR) as described in Diallo *et al.* (2015). By comparing the flow cytometry results with the SSR markers for the 59 mature trees, we concluded that polyploids could always be separated from diploids by the presence of more than two alleles per locus in at least 1 of the 8 loci (Appendix 1). Based on their SSR genotypes, we therefore assigned all 634 trees in the field trial to either diploid or polyploid status, but not distinguishing between tri-, tetra-, penta- or hexaploids.

2.5. Statistics

For the data from the field trial, a generalized linear analysis of variance was applied to test differences between the growth of diploid and polyploid trees in the trial. Trees that were assessed in 1995 but were not alive in 2012 were excluded, as their ploidy level was unknown. The analysis included the effects of ploidy level (diploid or polyploid), site of origin (Daiba, Diamenar, Kidira or Ngane) and block (30 levels) according to the following model:

$$Y=\text{Ploidy}+\text{Site of origin}+\text{Block}+\text{Error} \quad (1)$$

Where the effects of ploidy and site of origin were considered as fixed, block was considered as random, and the error followed a normal distribution with expectation zero. The average performance of diploids and polyploids was estimated as least square means from the analysis, *i.e.* averages corrected for systematic differences among provenances.

Data from the growth chamber experiment were analyzed in two steps. The first step was a model with the effects of descendance and blocks:

$$Y=\text{Descendance}+\text{Block}+\text{Error} \quad (2)$$

Where descendance was considered a fixed effect and the block was considered as random. From this model, we calculated the least square mean values of all variables for each descendance. Due to the limited number of samples for stomatal density and length, calculation of least square means was not possible and instead the mean values were calculated. Mean values were also calculated for seed traits. Next, as each descendance was either diploid or polyploid and originated from one of the four sites of origin, we applied the following model:

287 Y=Ploidy + Site of origin + Error (3)
288
289 Where Y denotes the least square means (or means) of the descendant families from model (2), and
290 ploidy and site of origin were considered as fixed effects.
291
292 All analyses were performed using the GLM procedure in the SAS 9.3 Software (SAS Institute Inc.
293 2014). Assumptions of variance homogeneity and normality of residuals were accepted for all the
294 studied characters based on visual inspection of residual plots. However, for the ratio between fresh
295 and dry weight in the growth chamber experiment a single outlier (Kidira family 7) was identified
296 and deleted from the data.
297
298
299 **3. Results**

300 **3.1. Ploidy level**

301 Both diploid and polyploid individuals were found among trees from all four origins, but at very
302 different frequencies: 136 of 164 trees (83%) originating from Ngane were polyploid, compared to 3
303 of 178 trees in Diamenar (2%), 14 of 146 trees in Daiba (10 %) and 16 of 117 trees in Kidira (14%).
304
305 Flow cytometric analyses of seedlings allowed separation between diploid, triploid, tetraploid,
306 pentaploid and hexaploid individuals corresponding to a mean 2C DNA content of 1.25 ± 0.02 pg,
307 1.96 ± 0.05 pg, 2.60 ± 0.03 pg, 3.19 pg and 3.83 ± 0.08 pg respectively. All offspring from diploid
308 mothers were diploid, while tetraploid mothers produced either offspring with the same ploidy level
309 (tetraploid) or higher levels (pentaploid, hexaploid and octaploid). Seeds from the same pod collected
310 on the tetraploid mother NG16_B19 gave rise to one tetraploid and one hexaploid seedling, while all
311 seedlings coming from the tetraploid mother DA15_B3 were hexaploid. Of 13 offspring tested from
312 the tetraploid parent NG10_B8, 11 were tetraploid, one was pentaploid and one was hexaploid; of the
313 16 plants examined from the tetraploid mother NG20_B3, one was octaploid and 15 were tetraploid.
314 Out of three seedlings tested from the triploid individual DA1_17, two were triploid and one was
315 tetraploid (Appendix 1).

316 **3.2. Growth differences between diploid and polyploid trees in the progeny trial**

317 The polyploid trees in the Dahra trial were significantly taller than the diploid trees 1 year after
318 planting in 1995. In 2012, after 18 years, differences between diploid and polyploid trees for both
319 height and trunk diameter were still significant, whereas crown diameter did not differ between
320 ploidy levels. The superiority of polyploids compared to diploids based on least square mean
321 estimates after 1 year was 18% for height, while 9% for height and 17% for trunk diameter after 18
322 years. The true differences between average of diploid and polyploid trees (i.e not corrected for
323 systematic effects of provenances) were substantially larger: 49% for height at age 1; and 15% and
324 24% for height and diameter respectively at age 18 (Table 1, Fig. 2).
325
326 **3.3. Phenotypic differences between diploid and polyploid seedlings in the drought stress test**
327
328 Polyploids differed significantly from diploids in seed length and width, initial fresh weight (at week
329 0 when transplanted to the boxes), leaflet length, stomatal density and length. Heights were similar
330 until weeks 10 and 11. Total fresh weight was borderline significant at the end of the trial, and the
331 fresh weight / dry weight ratio differed significantly between ploidy levels (Table 2, Fig. 2).
332

The morphological parameters showed that the polyploids tended to be larger than the diploids (Table 2). Seed length and width were 12% and 10% larger in polyploids compared to diploids, respectively. Polyploids had leaflets that on average were 19% longer than in diploid individuals, and the differences in stomatal density and length were pronounced: polyploid individuals were characterized by 54% wider stomata but with lower stomatal density (31% less) compared to diploids.

Prior to drought stress, the plant height was similar for both ploidy levels, but after 10 and 11 weeks (corresponding to 5 and 6 weeks of water deficit), the tetraploids has grown taller than diploids (22 and 28 %, respectively) (Table 2, Fig. 3). At the end of the trial, polyploid *A. senegal* seedlings had 60% larger fresh weight than diploids. Differences in dry weight were smaller because the fresh weight / dry weight ratio was 9% larger in polyploids than in diploids.

4. Discussion

4.1. Evolution of polyploidy in A. senegal

Our results showed that *A. senegal* can occur in more than two levels of ploidy, which supplement the results of Assoumane *et al*. (2013) and Odee *et al.* (2015) who reported diploid and tetraploid individuals. In our study, we found that diploids were most frequent followed by tetraploids. Pentaploids, triploids, and hexaploids were also present among seedlings although in small quantities. Based on small sample sizes, Odee et al. (2015) showed co-existence of ploidy types. This result is qualified by the present study, where we show that the frequency of polyploid individuals can vary significantly among natural populations in Senegal. Polyploids were dominant in the population from the central Senegal (Ngane) characterized by saline soils, whereas higher proportions of diploids were found in Diamenar (North), Daiba (North-east) and Kidira (South-east). Nevertheless, a few diploid individuals from the saline site (Ngane) were also identified and polyploids occurred in low frequency in non-saline areas (Kidira, Diamenar and Daiba sites).

Our data comparing the ploidy level of trees and their offspring indicated only very limited hybridization between cytotypes, even in a setup where diploid and polyploid trees were grown side by side in a field trial. Further, offspring from diploid mothers were always diploid suggesting that hybridization between cytotypes with diploid maternal trees must be very rare if at all possible. The higher ploidy levels occasionally found in offspring from tetraploid mothers on the other hand suggest that tetraploid mothers rather frequently produce some gametes that are unreduced. Hexaploid seedlings from tetraploid mothers may have been formed by unreduced egg cells (4n) sired by reduced pollen gametes from a tetraploid pollen donor. The identified pentaploid seedling could have been formed by a pollination event involving a hexaploid pollen donor from the field trial (however not among the trees that were tested with FCM). Alternatively the pentaploid seedling could originate from a fusion between an unreduced egg cell from the tetraploid mother and a reduced pollen gamete from a diploid pollen donor i.e. reflecting cytotype hybridiziation. In relation to this aspect, we found a single triploid mother DA1_B17 which might have been formed by cytotype hybridization in the previous generation. This triploid tree produced both triploid and tetraploid seedlings. Triploids have previously been reported when conspecific diploids and tetraploids co-occur in the same area as *e.g. Chameron angustifolum* (Husband, 2004) and may play an important role as a triploid-bridge allowing gene flow through mating between diploid and polyploid individuals (Henry *et al*., 2005) with recurrent polyploid formation in the population.

The frequency of triploid seedlings (2 out of 162 – both from a triploid mother) observed in our study is, however, low compared to the reported frequencies of triploids in species with mixed populations

(range of 2 - 22 %) as reviewed by Soltis *et al*. (2010). Also, no triploid seedlings were observed from either diploid or tetraploid mother trees, which support the presence of a significant reproductive barrier. Stebbins (1971) predicted that mating between ploidy levels is likely only from diploid fathers to polyploid mothers. Unidirectional mating from diploids to tetraploids is known from other species complexes such as *Sorghum* (diploid *Sorghum bicolor* and tetraploid *Sorghum halepense*) (Arriola & Ellstrand 1996), *Capsella rubella* (to the allotetraploid descendant *C. bursa-pastoris*) (Slotte *et al*., 2008), and *Arabidopsis arenosa* (Jørgensen *et al*., 2011). As pollen is aggregated in polyades in *A. senegal* and the stigmatic cavity is cup-shaped (Tandon et al., 2001) morphological size differences between cytotypes could also restrict hybridization. Additional detailed studies are needed to clarify the strength of the reproductive barriers between cytotypes of *A. senegal*, and if pollination is always unidirectional under natural conditions.

4.2. Adaptive potential and evolutionary success of polyploids

Polyploid trees often occupy drier habitats than their diploid relatives (e.g. Li et al., 1996, Pettigrew et al., 2012). Experiments have shown different performance of diploids and polyploids under drought stress, but are limited by their short duration and experimental setup (Li et al., 1996, Li et al., 2009). Unfortunately, there is very limited evidence from long-term field trials on the relative performance of trees with different ploidy levels. The faster growth of polyploid *A. senegal* in our study is to our knowledge the first observation of superiority of mature natural polyploid trees in a field trial and indicates that at least under some conditions, trees with high ploidy levels will have an adaptive advantage. Although the effects of ploidy level and origin were to some extent confounded due to the observed unequal distribution of polyploids, our statistical analysis showed that the positive effect of being polyploid remained even when accounting for the effect of origin.

The field trial represents relatively dry conditions close to the Northern limit of distribution of the species towards the Sahara desert. Since the relative performance of diploid and polyploid *A. senegal* has not been investigated under wetter conditions, it is not possible to conclude whether the better growth is found only under dry conditions or indicates a general superior performance.

Still, the growth chamber experiment showed that polyploid plants only grew faster than diploids after the plants were subjected to water stress. The limited number of polyploid plants did not allow us to include a control treatment where seedlings were continuously raised without water stress, and we hence do not separate potential ontogenetic effects from effects of drought. The question of whether the adaptive advantage of polyploids is limited to dry conditions or applies over a broader range of environments therefore remains unresolved. Reciprocal experiments ('optimal' versus a single abiotic stress factor) to test the relative performance of di- and polyploids under different stress situations are needed to conclude whether polyploids are generally superior or if it is only the case under dry conditions (*cf.* Soltis *et al*., 2010). Investigations in other heteroploid tree species or species complexes are needed to reveal if the observed effects of polyploid in *A. senegal* reflect a so-far undiscovered pattern in trees species growing under stressful conditions in Sahel.

Polyploidy is often associated with a difference in plant phenotype (e.g. increase in cell size, enlarged floral structure, pollen, stomata and robust stems) when compared to the diploid relatives (Ramsey & Schemske 1998; Madlung, 2013). In our study, we found the first evidence of phenotype differentiation between cytotypes in *A. senegal*, as seeds, leaflets and stomata were larger and stomatal densitites were smaller in tetraploids of *A. senegal*. This confirms results reported for other tree species, such as *Adansonia digitata* (Pettigrew *et al*., 2012), *Betula papyrifera* (Li *et al*., 1996), *Acacia mangium* (Harbard *et al*., 2012) and *Acacia maernsii* (Beck *et al*., 2003). Studies based on neopolyploids have shown that many of these polyploid characteristics are directly linked to

increased genome size (*e.g.*, Harbard *et al*., 2012). It is unknown whether the size differences in *A. senegal* are caused by increased genome size, increased genetic diversity or a combination of both.

The observed phenotypic differences are likely to lead to differences in physiology, as gas movement in and out of leaves is affected by leaf size, size and distribution of stomata. It has been hypothesized that the fewer, but larger stomata observed in polyploids can change stomatal conductance and confer increased water use efficiency to polyploids under drought stress (*e.g.* Li *et al*., 1996; Li *et al*., 2009; Pettigrew *et al*., 2012). Assuming that width and depth of the stomatal pore is proportional to the length, it can easily be estimated following Franks & Farquahar (2001) and Franks & Beerling (2009)) that stomatal conductance is expected to be 5% larger in polyploids than diploids. On the other hand, estimates based on leaf dimensions (Nobel, 2009) suggest that leaf boundary layer conductance will be reduced by 9% in polyploids compared to diploids. Hence the effects of leaf size tend to negate effects of changed stomatal size and density, and the expected overall effects of ploidy level on water use efficiency are therefore unclear. Detailed anatomical studies, coupled with assessments of gas exchange on trees with different levels of ploidy will be important in order to infer on potential mechanisms behind the putative selective advantage of polyploidy in *Acacia senegal*. For example, in the herbaceous perennial *Chamerion angustifolium*, Maherali *et al*. (2009) found that tetraploids characterized by large stomata and wide xylem vessels did not differ from diploids in stomatal conductance and gas exchange when grown under drought conditions. However, increased hydraulic conductivity was believed to cause increased drought resistance of tetraploids in this species.

Another observed phenotypic difference with potential physiological consequences is the larger fresh to dry weight ratios of polyploids compared to diploids (Table 2) causing the ploidy levels to differ almost significantly in fresh weight, but not in dry weight. High water contents indicate either a larger capacity for osmotic adjustment or an increased elasticity of cell walls (Verslues et al. 2005), and Li et al. (1996) suggested that polyploids might have a larger ability to adjust their osmotic potential under drought stress. If this is indeed the case, it may explain part of the better performance of polyploids in *A. senegal*.

In conclusion, the co-existence of different ploidy levels in natural populations was confirmed while the pattern of segregation supports that gene flow between cytotypes is limited. Our results document increased growth of polyploid *A. senegal* both in the field trial and under growth chamber conditions, but it remains to be verified if superiority of polyploids is expressed only under relatively dry conditions or applies more generally.

Author contributions

AD, LN, EK and AR conceived the ideas; AD, LN and KP collected the data; AD, EK and AR carried out the statistical analyses; all authors analyzed and interpreted the data, and all authors contributed to writing of the paper.

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Conflict of interest

The authors declare no conflict of interest.

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690 **Figure legends**

691
692 **Figure 1.** Location of the progeny trial (Dahra) and the four tested provenances of *Acacia senegal* in
693 Senegal
694
695 **Figure 2.** Performance of polyploids expressed in percent of diploid performance. The vertical dotted
696 line (100%) denote diploids. Error bars denote the 95% confidence limits of differences between
697 polyploids and diploids.

698 **Figure 3.** Variation in height between diploid (filled circles) and polyploid seedlings (open circles) of
699 *Acacia senegal* under drought stress conditions. The arrow indicates onset of the drought stress,
700 while error bars denote SD.
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Tables

Table 1. F-tests for significance in growth traits between ploidy levels and provenances in the field trial and LS estimated averages of diploid and polyploid trees

	Ploidy level			Provenance			Ploidy (Average performance)			
Traits	*Df;*	*F*	*P>F*	*Df;*	*F*	*P>F*	Diploid (LSmean)	Polyploid (LSmean)	Diploid (Mean)	Polyploid (Mean)
Height 1995 (cm)	1; 29	5.96	0.015	3; 29	14.61	0.001	38.2(*1.18*)	45.0(*2.29*)	38.2 (16.3)	57.0(22.3)
Height 2012 (m)	1; 29	9.52	0.002	3; 29	4.24	0.006	4.52(*0.05*)	4.92(*0.11*)	4.57(0.05)	5.25(0.07)
Diameter 2012 (cm)	1; 29	17.29	0.001	3; 29	3.64	0.013	11.86(*0.19*)	13.92(*0.41*)	11.57(0.16)	14.34(0.28)
Crown diameter 2012 (m)	1; 29	0.06	0.804	3; 29	1.76	0.152	5.50(*0.07*)	5.55 (*0.14*)	5.40(0.06)	5.61(0.09)

Least square means (LS means), Simple average (Mean) and standard errors (*SE*) are estimated from the analysis of variance. *Df*: Degrees of freedom; *F*: *F*-value.

Table 2. F-tests for significance of morphological differences between ploidy levels and provenances of *Acacia senegal* in the growth chamber trial.

	Ploidy level			Ploidy LS means	
Traits	Df; Error	F	P>F	Diploid	Polyploid
Seed traits					
Number of seeds per pod	1; 55	0.34	0.56	4.4 (*0.2*)	4.1 (*0.5*)
Seed length (mm)	1; 49	13.6	0.0006	7.7 (*0.1*)	8.7 (*0.2*)
Seed width (mm)	1; 49	6.5	0.01	7.8 (*0.1*)	8.6 (*0.2*)
Germination rate (%)	1; 55	0.94	0.34	89 (*3*)	*95* (*6*)
Growth and morphology at 87 % field capacity					
Initial total fresh weight (g)	1; 49	12.2	0.001	0.17 (*0.01*)	0.27 (*0.02*)
No. leaves	1; 48	0.02	0.89	16.0 (*1.0*)	16.3 (*2.2*)
No. branches	1; 48	0.00	0.99	6.2 (*0.4*)	6.2 (*0.8*)
Leaf length (cm)	1; 31	0.03	0.87	1.9 (*0.1*)	1.9 (*0.2*)
Leaflet length (mm)	1; 31	5.1	0.03	6.1 (*0.2*)	7.3 (*0.5*)
Leaflet width (mm)	1; 31	2.0	0.17	1.8 (*0.1*)	2.1 (*0.1*)
Stomatal density (mm^{-2})	1; 31	13.7	0.0008	204 (*7*)	139 (*15*)
Stomatal lenght (µm)	1; 31	57	<0.0001	46 (*1*)	71 (3)
Growth at 47 % field capacity					
Height at week 8 (cm)	1; 48	0.97	0.33	15.9 (*0.6*)	17.6 (*1.4*)
Height at week 9 (cm)	1; 48	2.4	0.13	17.2 (*0.7*)	20.0 (*1.5*)
Height at week 10 (cm)	1; 48	4.1	0.05	18.1 (*0.7*)	22.2 (*1.6*)
Height at week 11 (cm)	1; 48	6.3	0.02	19.1 (*0.8*)	24.5 (*1.7*)
Total fresh weight (g)	1; 48	3.6	0.06	1.0 (*0.1*)	1.6 (*0.2*)
Total dry weight (g)	1; 48	1.6	0.21	0.33 (*0.03*)	0.44 (*0.07*)
Fresh weight/dry weight ratio	1; 47	5.4	0.03	3.30 (*0.05*)	3.60 (*0.10*)

Least square means (LS means) and standard errors (*SE*) are given for the two ploidy levels. Df: Degrees of freedom and F: F-value.

Appendix 1. Ploidy levels in a sub-set (76 parents) of *Acacia senegal* in the progeny trial and in their offspring revealed by eight polymorphic microsatellites and flow cytometry (FCM) respectively

Parent ploidy levels								Offspring ploidy levels					
Provenance	Family	Ind.	No. loci with 1-2 alleles	No. loci with more than 2 alleles	FCM on twig	No. Pods tested	No. offspring tested	Diploid (2n)	Triploid (3n)	Tetra (4n)	Penta (5n)	Hexa (6n)	Octo (8n)
Ngane	NG4	*B14	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG4	B16	4	4	Na	5	11	-	-	10	-	1	-
Ngane	**NG7**	***B22**	**8**	**0**	**Diploid**	**1**	**2**	**2**	-	**-**	-	-	-
Ngane	NG10	*B8	7	1	Tetraploid	6	13	-	-	11	1	1	-
Ngane	**NG11**	***B17**	**8**	**0**	**Diploid**	**1**	**2**	**2**	-	**-**	-	-	-
Ngane	NG14	B1	5	3	Na	5	12	-	-	11	-	1	-
Ngane	NG14	*B2	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG15	*B16	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG16	B3	5	3	Na	4	9	-	-	9	-	-	-
Ngane	NG16	*B19	4	4	Tetraploid	1	2	-	-	1	-	1	-
Ngane	NG17	*B8	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG18	*B25	4	4	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG19	B1	4	4	Na	4	8	-	-	6	-	2	-
Ngane	NG19	* B24	4	4	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG20	*B3	4	4	Tetraploid	6	16	-	-	15	-	-	1
Ngane	NG21	* B4	4	4	Tetraploid	6	13	-	-	12	-	1	-
Ngane	NG22	*B6	5	3	Tetraploid	1	2	-	-	2	-	-	-
Ngane	NG25	*B30	4	0	Diploid	4	10	10	-	-	-	-	-
Ngane	NG26	*B17	4	4	Tetraploid	1	2	-	-	2	-	-	-
Diamenar	DIA2	B1	8	0	Na	1	6	6	-	-	-	-	-
Diamenar	DIA2	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA6	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA6	B3	8	0	Na	1	4	4	-	-	-	-	-
Diamenar	DIA7	*B27	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA8	*B21	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA11	* B5	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA13	*B26	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA14	*B6	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA15	B8	8	0	Na	1	2	2	-	-	-		-
Diamenar	DIA15	*B21	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA17	*B17	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA18	B4	8	0	Diploid	1	2	2	-	-	-	-	-
Diamenar	DIA20	*B23	8	0	Na	1	2	2	-	-	-	-	-

Diamenar	DIA22	*B6	8	0	Na	1	3	3	-	-	-		-
Diamenar	DIA22	*B14	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA26	*B2	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA27	*B5	8	0	Na	1	2	2	-	-	-	-	-
Diamenar	DIA29	*B5	8	0	Na	1	2	2	-	-	-	-	-
Daiba	DA1	B17	7	1	Triploid	1	3	-	2	1	-	-	-
Daiba	DA1	*B19	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA2	*B5	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA4	*B12	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA4	*B22	7	1	Na	1	3	-	-	3	-	-	-
Daiba	DA6	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA7	*B1	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA8	*B17	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA13	*B9	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA15	*B3	7	1	Tetraploid	1	2	-	-	-	-	2	-
Daiba	DA15	B11	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA16	*B15	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA17	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA18	B2	8	0	Diploid	1	4	4	-	-	-	-	-
Daiba	DA18	B5	8	0	Diploid	1	1	1	-	-	-	-	-
Daiba	DA19	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Daiba	DA20	*B13	8	0	Diploid	1	4	4	-	-	-	-	-
Daiba	DA25	*B8	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA25	B12	8	0	Diploid	1	3	3	-	-	-	-	-
Daiba	DA26	B2	8	0	Diploid	1	5	5	-	-	-	-	-
Daiba	DA26	B9	8	0	Diploid	1	3	3	-	-	-	-	-
Kidira	K1	*B3	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K3	*B2	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K4	*B23	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K5	*B7	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K7	*B8	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K8	*B7	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K9	*B5	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K14	*B6	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K16	*B15	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K17	B14	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K20	*B9	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K21	B13	8	0	Diploid	1	5	5	-	-	-	-	-
Kidira	K21	*B22	4	4	Tetraploid	1	2	-	-	2	-	-	-
Kidira	K22	*B9	8	0	Diploid	1	2	2	-	-	-	-	-
Kidira	K23	B3	6	2	Na	1	Na	Na	-	Na	-	-	-

Kidira	K23	B6	8	0	Diploid	1	4	4	-	-	-	-	-
Kidira	K25	*B16	4	4	Tetraploid	1	2	-	-	2	-	-	-

In bold: The pure diploid Ngane families (NG7 & NG11). Dark grey: triploid mother producing both triploid and tetraploid offspring. Light grey: Families with mixed ploidy levels. Individuals marked with * were used in the drought stress trial. Na: Not assessed.

Figure 01.JPEG

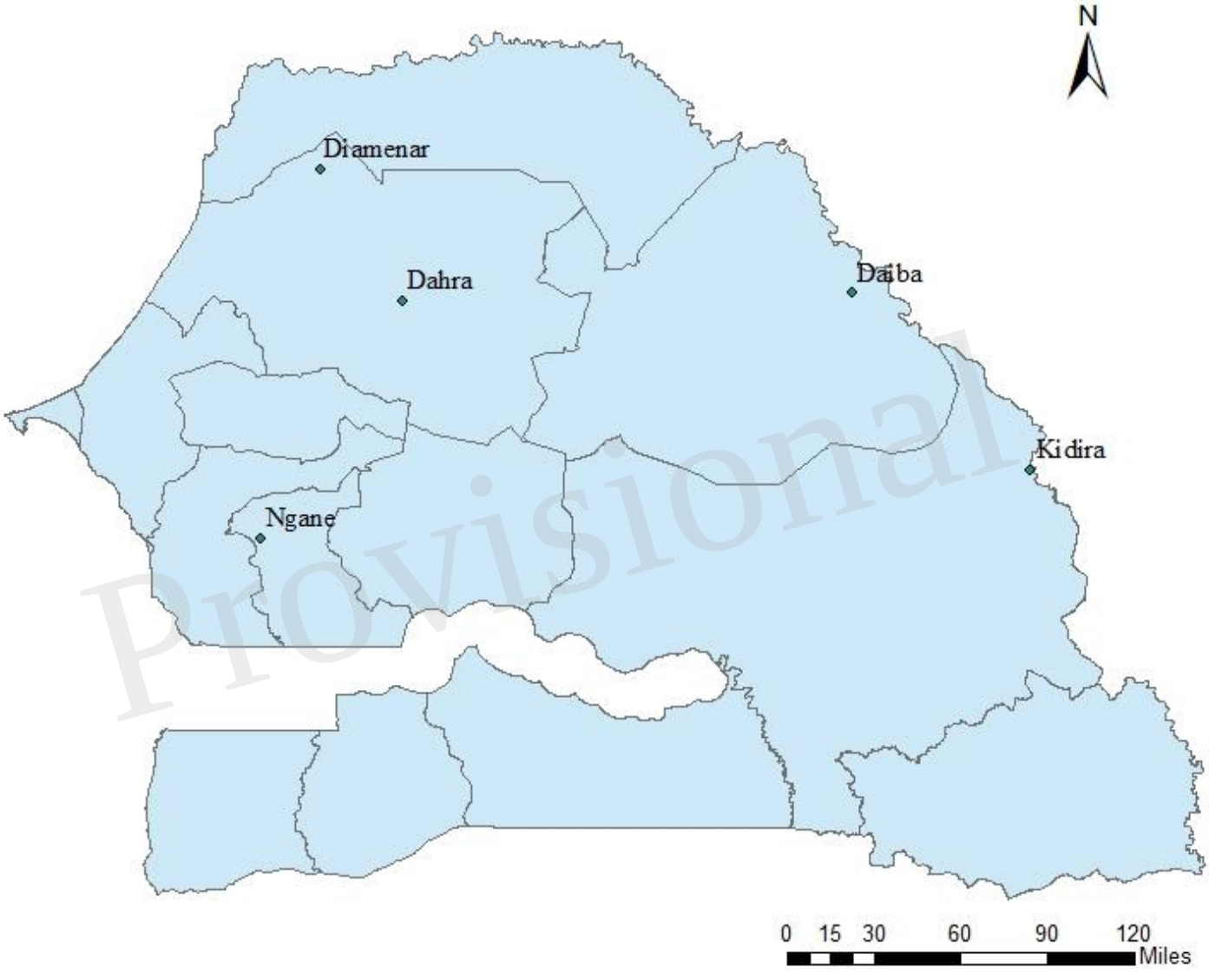

Figure 02.TIF

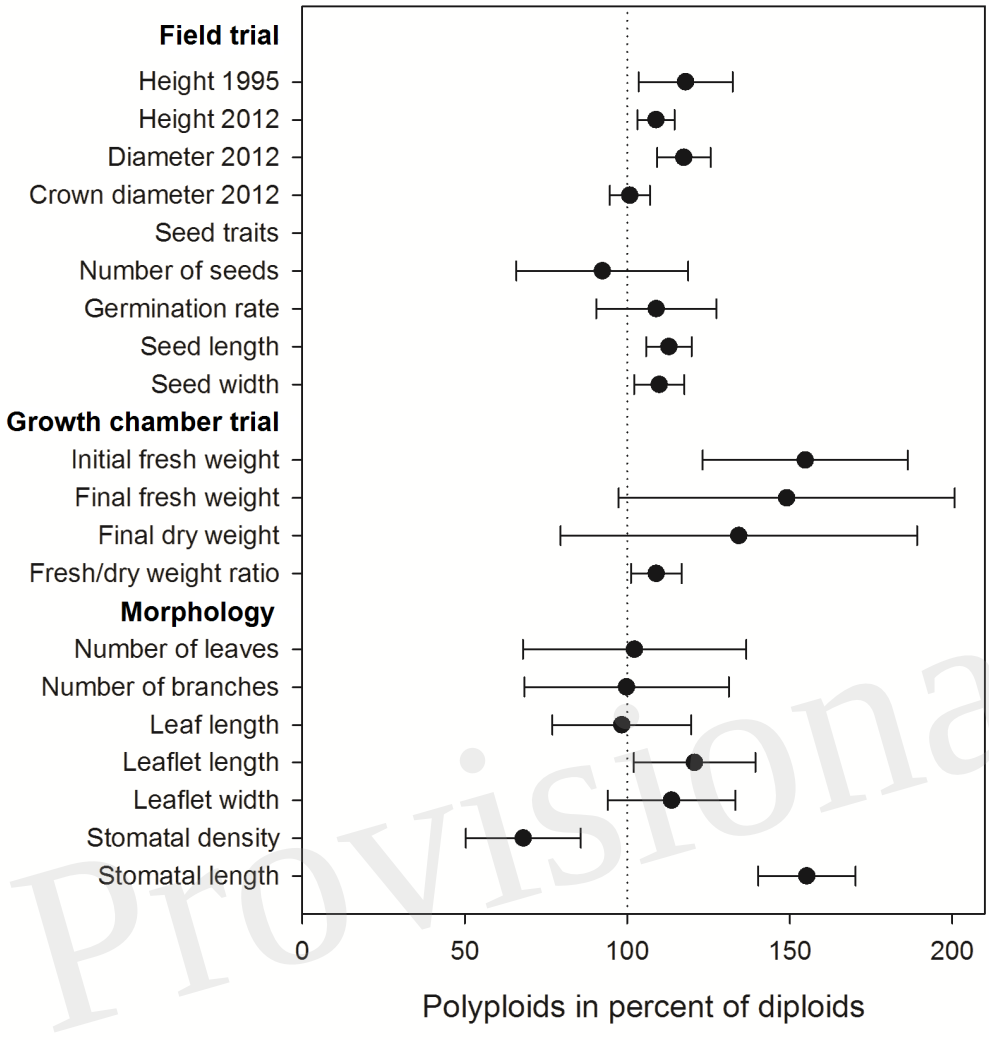

Figure 03.JPEG