

Cypress Surrogate Mother Produces Haploid Progeny From Alien Pollen

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ABSTRACT

Although most living organisms reproduce sexually, some have developed a uniparental reproduction where the embryo usually derives from the female parent. A unique case of paternal apomixis in plants has been recently reported in *Cupressus dupreziana*, an endangered Mediterranean conifer. This species produces unreduced pollen that develop into all-paternal embryos within the seed tissues. We analyzed seedlings produced by open-pollinated *C. dupreziana* seed trees using morphological descriptors, ploidy levels assessed through flow cytometry, and AFLP genetic diversity. *In situ C. dupreziana* seed trees (from Algeria) produced only diploid *C. dupreziana* progeny. In contrast, only one-third of the progeny produced by *ex situ C. dupreziana* seed trees planted in French collections were similar to *C. dupreziana* seedlings; the other progeny were haploid or diploid *C. sempervirens* seedlings. These results demonstrate that *C. dupreziana* ovules allow for the development of all-paternal embryos from pollen produced by another species, *C. sempervirens*. Thus, the *in planta* androgenesis is achieved through the combination of the embryogenic behavior of pollen grains and the ability of seed tree ovules to act as a surrogate mother. This phenomenon offers a unique opportunity to produce, by natural means, highly valuable material for genetic studies and selection of sterile cultivars.

SEXUAL reproduction is the general rule in higher plants and animals. More than 99% of multicellular eukaryotes reproduce sexually (BELL 1982; BARTON and CHARLESWORTH 1998; WEST *et al.* 1999). The sexual cycle follows the alternation of haploid and diploid cells delimited by meiosis and syngamy, two events that modify the nuclear chromosome number (VRIJENHOEK 1998). However, in some asexual species or lineages, the diploid embryo is of uniparental origin. In plants, at least 300 species in 40 angiosperm families (mainly Poaceae, Asteraceae, Rosaceae, and Rutaceae) exhibit patterns of apomixis where the embryo is derived solely from maternal tissue, either a somatic cell or a gamete with the somatic chromosome number (unreduced gamete) (VIELLE CALZADA *et al.* 1996). In rarer cases, the parthenogenetic development of a reduced gametophyte produces a haploid embryo (FOROUGH-WEHR and WENZEL 1993). This last feature, also called non-recurrent apomixis, occurs naturally in some female gametophytes. It is also intensively used in breeding programs through the *in vitro* culture of male gametophytes (androgenesis). Diploid homozygous genotypes can then be obtained through natural or artificial doubling of the chromosome number.

Until recently, apomixis had been reported only in Angiosperms. The highly endangered Mediterranean conifer, *Cupressus dupreziana* A. Camus (or Tassili cypress), is to our knowledge the first plant species where *in planta* "paternal apomixis" (*i.e.*, embryogenic development of diploid pollen in seed tissues) was hypothesized (PICHOT *et al.* 2000). This monoecious species is the most notable tree species of the mountainous region of the Tassili N'Ajjer plateau in the central Algerian Sahara desert (BALACHOWSKY 1955). The natural population today is reduced to 233 trees spread over 1200 km² on the southwestern border of the Tassili N'Ajjer plateau (≥ 1500 m above sea level) (ABDOUN and BEDDIAF 2002). Low accessibility due to high cliffs has contributed to protecting these trees from traditional harvesting for timber supply in Djanet and Ghat oases. In more accessible regions, overexploitation led to the extinction of the populations. Both extreme climatic conditions (the mean annual rainfall averages 30 mm) and a high human pressure may explain the very poor natural regeneration observed. The species produces unreduced diploid pollen whose embryogenic ability was demonstrated in interspecific pollinations; the controlled pollination of ovules of *Cupressus sempervirens* L. (or Mediterranean cypress) by pollen of *C. dupreziana* produces diploid embryos of *C. dupreziana* in *C. sempervirens* seeds (PICHOT *et al.* 2001). Reciprocally, we demonstrate here that the *C. dupreziana*

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TABLE 1

Classification of cypress progeny produced by open-pollinated *C. dupreziana* seed trees on the basis of their morphological and genetic characteristics

Geographic origin	No. of seed trees	No. of seedlings	Morphology ^a	Ploidy ^b	AFLP profile ^c	Figure
<i>In situ</i> Tassili N'Ajjer, Algeria	6	38	Cd	2C	Cd	Figure 1A
<i>Ex situ</i> southeastern France	26	54	Cd	2C	Cd	Figure 1A
		32	Cs	2C	Cs	Figure 1B
		74	Cs	1C	Cs	Figure 1C

^a Cd, *C. dupreziana*-like with long and sharp juvenile leaves and light-green foliage; Cs, *C. sempervirens*-like with short juvenile leaves and dark-green foliage.

^b Relative DNA content revealed by flow cytometry as compared to diploid (2C) somatic tissues used as the control.

^c Based on a discriminant analysis of cypress species using 32 AFLP markers. Cd, *C. dupreziana* profile; Cs, *C. sempervirens* profile.

surrogate mother ability makes it possible to develop haploid embryos from reduced pollen produced by another cypress species. To our knowledge, this is the first report of natural *in planta* androgenesis in plants.

MATERIALS AND METHODS

Seedling production: Seed lots were collected from 6 open-pollinated *in situ* *C. dupreziana* trees (Tassili N'Ajjer desert, Algeria) and from 26 open-pollinated *C. dupreziana* trees from four southeastern France, *ex situ*, cypress collections (Carpentras, 1 seed tree; Estérel, 18 seed trees; Montpellier, 5 seed trees; and Ruscas, 2 seed trees. *Ex situ* collections contained other cypress species that may have contributed to pollination. Seeds were sown in petri dishes and germinating seeds were transplanted in 400 cm³ containers and cultivated in nursery.

Morphological observations and ploidy level: Number of cotyledons, length and shape of juvenile leaves, and foliage color were recorded on 4-month-old seedlings to discriminate *C. dupreziana* and *C. sempervirens* phenotypes. Seedling ploidy level was assessed by flow cytometry. Nuclei of fresh somatic tissues were stained with 4',6-diamidino-2-phenylindole and analyzed using a PARTEC Ploidy Analyser PA following the protocol described by PICHOT and EL MAËTAOUI (2000).

The effect of seed tree geographic origin on the proportion of the different phenotypes and ploidy levels of the progeny was tested using a generalized linear model with the logit link function (COLLETT 1991). Statistical analyses were performed with the R software (R DEVELOPMENT CORE TEAM 2004).

Genetic diversity: To infer the genetic origin of the seedlings, we compared their genetic patterns revealed by amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995) to those of 14 cypress species available from INRA (French National Institute for Agricultural Research) collections: *C. sempervirens* (7 samples), *C. atlantica* (5), *C. dupreziana* (43), *C. arizonica* (5), *C. goveniana* (5), *C. forbesii* (4), *C. lusitanica* (5), *C. macnabiana* (2), *C. macrocarpa* (5), *C. benthamii* (5), *C. funebris* (5), *C. cashmeriana* (5), *C. chengiana* (5), *C. torulosa* (5). AFLP analysis was conducted using the protocol (slightly modified) of ZABEAU and VOS (1993). DNA was digested with *EcoRI* and *MseI* restriction enzymes. Amplifications were performed using a Gradient 96 Stratagene (La Jolla, CA) Robotcycler. Selective amplifications were performed using *EcoRI* + ACG and *MseI* + CAG primer pairs. Silver staining was conducted according to the procedure (slightly modified) described by CRESTE *et al.* (2001). The presence/absence of AFLP marker bands was scored for all individuals.

The individual AFLP profiles of the seedlings were plotted as supplemental points on the plane defined by the first two axes of the discriminant analysis performed on the genetic profiles of the cypresses used as the control (14 species). The discriminant multivariate analysis was performed using the Multidim package (CARLIER and CROQUETTE 2002) within the R software.

RESULTS

Characteristics of control cypress species used for the identification of the progeny are described first. Progeny features are then reported and summarized in Table 1.

Morphological characteristics and ploidy level of the controls: The embryos of Mediterranean and Asian species have two or, more rarely, three cotyledons while American species have three to five cotyledons. Juvenile leaves of *C. dupreziana* seedlings are longer and sharper than those of *C. sempervirens*. *C. dupreziana* foliage has a lighter green color. All cypress species used as the control were diploid and exhibited similar DNA contents in flow cytometry measurements.

AFLP pattern of the controls: Genetic diversity was estimated with 32 polymorphic AFLP markers. The discriminant analysis of AFLP profiles for the control species (106 individuals) clearly discriminated their three main geographic origins—America, Asia, and the Mediterranean basin—and also led to a clear differentiation among Mediterranean species. Moreover, five AFLP bands were diagnostic markers for *C. sempervirens* (one band) and *C. dupreziana* (four bands). A nuclear biparental inheritance is usually reported for AFLP markers (LERCETEAU and SZMIDT 1999; KAKEHI *et al.* 2005). A nuclear inheritance was also demonstrated for 6 of our 32 markers by analyzing segregation of AFLP bands in progeny produced by *C. sempervirens*-controlled crosses (data not shown). The 26 remaining markers were not analyzable due to the lack of polymorphism.

Characteristics of the progeny from *in situ* *C. dupreziana* seed trees (Table 1): All 38 seedlings produced by the six *in situ* *C. dupreziana* seed trees had two

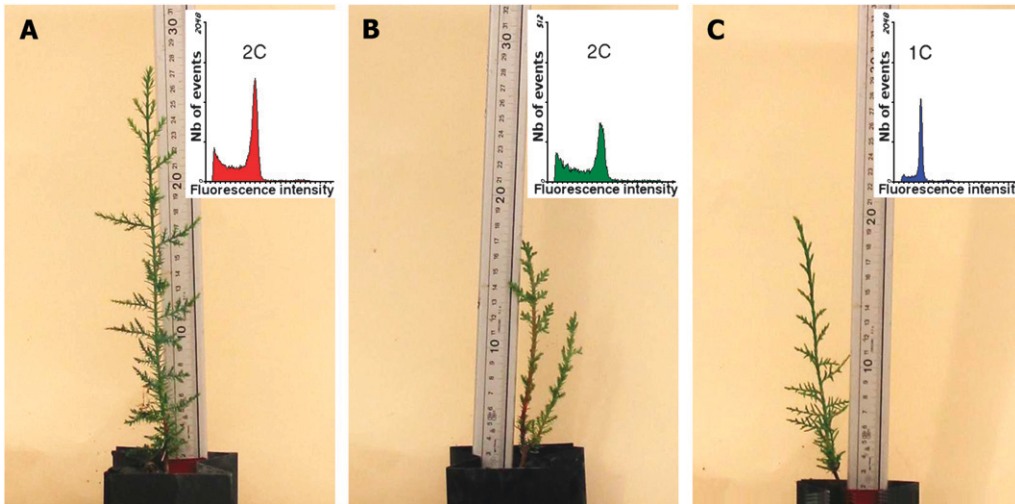


FIGURE 1.—Morphology and ploidy of the progeny produced by open-pollinated *C. dupreziana* seed trees. (A) Diploid *C. dupreziana* phenotype from *in situ* and *ex situ* seed trees. (B) Diploid *C. sempervirens* phenotype from *ex situ* seed trees. (C) Haploid *C. sempervirens* phenotype from *ex situ* seed trees.

cotyledons and exhibited a *C. dupreziana* phenotype (Figure 1A). Their relative fluorescence intensity peaks corresponded to those of somatic diploid tissues used as the control. AFLP patterns of these seedlings matched those of *C. dupreziana* controls.

Characteristics of the progeny from *ex situ* *C. dupreziana* seed trees (Table 1): Within the 160 seedlings produced by the *ex situ* open-pollinated *C. dupreziana* seed trees, two contrasted phenotypes were observed. A typical *C. dupreziana*-like phenotype was observed for 54 seedlings (Figure 1A) and a *C. sempervirens*-like phenotype was observed for 106 seedlings. *C. dupreziana*-like seedlings had two cotyledons except for one with three cotyledons. They were diploid and had AFLP profiles like *C. dupreziana* (Figure 2).

Most of the *C. sempervirens*-like seedlings had reduced growth and sometimes exhibited an unusual bushy shape. About one-third (32 of 106) of these seedlings were diploid (Figure 1B). They had two cotyledons, except for one with three cotyledons. The remaining two-thirds produced peaks of fluorescence intensity corresponding to half the diploid DNA amount and were consequently considered as haploid (Figure 1C). All haploid seedlings had two cotyledons. Irrespective of their ploidy

levels, all *C. sempervirens*-like seedlings exhibited AFLP profiles that matched those of the *C. sempervirens* control trees (Figure 2). Almost all *ex situ* *C. dupreziana* seed trees produced haploid seedlings. Only five seed trees, with a very low number of seedlings (four with one and one with three), produced only diploid progeny.

The proportion of *C. sempervirens*-like progeny *vs.* *C. dupreziana*-like progeny varied strongly from one site to the other, but the proportion of haploid seedlings within the *C. sempervirens*-like progeny was less variable (Table 2).

DISCUSSION

All-paternal genetic origin of the seedlings: The morphological and genetic characteristics of the *C. sempervirens*-like progeny produced by *ex situ* *C. dupreziana* seed trees indicate a strictly paternal origin of these seedlings. This result confirms the ability of cypress pollen to produce an embryo within the seed tissues of another cypress species but without genetic contribution of the seed tree, as reported when *C. sempervirens* seed trees were pollinated by *C. dupreziana* pollen (PICHOT *et al.* 2001). The androgenetic process occurred from

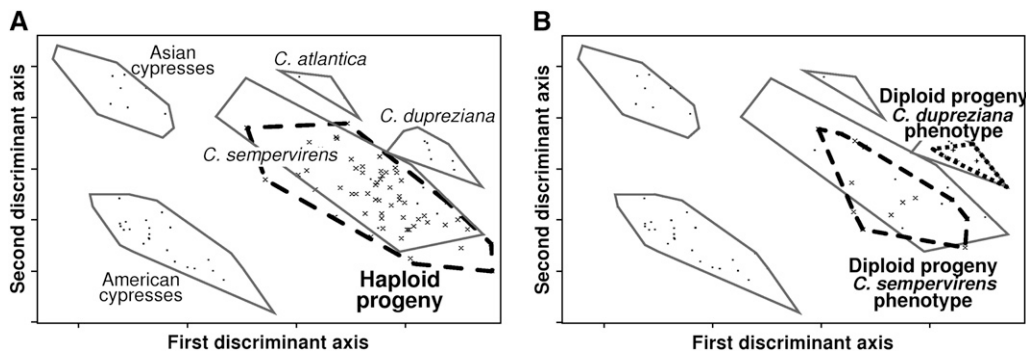


FIGURE 2.—Investigation of the genetic origin of progeny using AFLP markers. Only progeny produced by *C. dupreziana* seed trees planted in *ex situ* collections were studied. The individual genetic profiles were plotted as supplemental points on the plane defined by the first two axes of a discriminant analysis of cypress species used as a control (the three Mediterranean species

C. sempervirens, *C. atlantica*, and *C. dupreziana*; one American cypress group; and one Asian cypress group). All haploid progeny (A) were assigned to *C. sempervirens*. For diploid progeny (B), the genetic assignment either to *C. dupreziana* or to *C. sempervirens* was fully consistent with the classification based on the morphological type (Table 1, Figure 1).

TABLE 2

Variability of the proportion of the different phenotypes and ploidy levels according to the geographic origin of the seed trees

	<i>Ex situ C. dupreziana</i> collection				P-value ^a
	Carpentras	Estérel	Montpellier	Ruscas	
No. of seedlings	17	84	41	18	
% of <i>C. sempervirens</i> -like progeny	41	90	34	50	4.6×10^{-11}
% of haploid cypresses within <i>C. sempervirens</i> -like progeny	100	70	50	78	5.2×10^{-2}

All seed trees were open-pollinated *C. dupreziana* cypresses planted in the *ex situ* collection in southeastern France.

^aProbability of the observed proportions assuming no site effect and computed from a logit generalized linear model.

unreduced diploid pollen of *C. dupreziana* (PICHOT and EL MAËTAOUI 2000; EL MAËTAOUI and PICHOT 2001) and all the progeny from *C. sempervirens* seed trees were diploid. In contrast, *C. sempervirens* produces haploid pollen grains and both haploid and diploid *C. sempervirens*-like progeny were produced from *ex situ*, open-pollinated *C. dupreziana* seed trees. The haploid progeny would thus directly derive from the embryogenic development of the microgametophytes, initiated by haploid *C. sempervirens* pollen grains in a way similar to that of the diploid *C. dupreziana* microgametophytes, which produce diploid androgenic embryos. We demonstrate that the diploid progeny were pure *C. sempervirens* genotypes. However, an origin from unreduced gametes seems unlikely since no diploid nuclei were observed in *C. sempervirens* mature pollen (PICHOT and EL MAËTAOUI 2000). They could derive either from the fusion of two male gametes or from the early natural diploidization of the haploid embryo. The reduced growth and the abnormalities observed in most of the diploid *C. sempervirens*-like seedlings support the diploidization hypothesis or the fusion of two genetically identical gametes (*i.e.*, deriving from the same pollen grain). Indeed, it is well known that homozygosity produces inbreeding depression in allogamous species (CHARLESWORTH and CHARLESWORTH 1987). Complementary genetic analyses using codominant nuclear markers are planned to check for the putative homozygosity of these progeny.

Species compatibility: Although *C. dupreziana* seed trees planted in French cypress collections were surrounded by many other cypress species, all the seedlings deriving from alien pollen were *C. sempervirens* trees. It must be stressed that no seedlings from American cypress species were observed although many *C. dupreziana* seed trees were close to *C. arizonica* cypresses and that their flowering periods clearly overlap (PICHOT 2000). The large genetic distance between these species as compared to the proximity between the two Mediterranean species may explain this feature. According to recent studies, the genus Cupressus is paraphyletic

(LITTLE *et al.* 2004; LITTLE 2006). Cypresses from the New World, now recombined into the genus Callitropsis, and cypresses from the Old World (Mediterranean and Asian species), keeping the genus name Cupressus, do not share the same phylogenetic origin.

Significant variation in the proportion of *C. sempervirens*-like *vs.* *C. dupreziana*-like progeny observed among sites (Table 2) may be due to the relative contribution of the two species to the pollen cloud. Indeed, the highest rate of *C. sempervirens*-like progeny was observed in seeds collected from the Estérel plantation where *C. dupreziana* seed trees are the smallest and have a very low pollen production.

Conditions for natural androgenesis: Although the production of all-maternal progeny by apomixis or parthenogenesis is a rather frequent phenomenon, the production of all-paternal progeny has been very rarely reported (MCKONE and HALPERN 2003). In animals, it has been reported to occur naturally in freshwater clams (*Corbicula* genus) (KOMARU *et al.* 1998) and in interspecific hybrids of the Sicilian stick insect *Bacillus rossius-grandii* (TINTI and SCALI 1995). It was also observed in *Drosophila melanogaster* mutants (KOMMA and ENDOW 1995) and following fish egg irradiation (ARAKI *et al.* 1995). To our knowledge, *C. dupreziana* is the only plant in which progeny are produced by the apomictic development of pollen grains. The scarcity of androgenic cases reported compared to the amount of gynogenic case reports may be explained not only by the probable evolutionary dead end of this reproductive process but also by the difficulty in detecting it (MCKONE and HALPERN 2003). The male component ability to produce an embryo without female gamete contribution may not be so rare. In fact, it is intensively used in Angiosperms to produce haploid genotypes from *in vitro* culture of immature anthers. Thus, favorable growth conditions combined with a lack of syngamy opportunity may often lead to androgenesis. Such conditions occur naturally in the *C. dupreziana* × *C. sempervirens* cross-pollination system due to the production of unreduced diploid male and female spores

in *C. dupreziana* (PICHOT *et al.* 1998; PICHOT and EL MAËTAOUI 2000).

Consequences for *C. dupreziana* genetic preservation:

Natural production of *C. sempervirens* cypresses by *C. dupreziana* seed trees is an unusual type of genetic pollution although it is, according to our observations, restricted to seed admixture and does not include hybridization. The apomictic reproductive process protected the species from extinction for millennia and today prevents it from interspecific hybridization. This feature greatly facilitates the genetic conservation of the Tassili cypress and its safe propagation by seeds. However, only seeds collected from *C. dupreziana* seed trees that are very distant from other cypress species can be considered as pure *C. dupreziana* seeds. In other cases, seedling phenotype has to be checked to eliminate all non-*C. dupreziana* individuals, even if these alien seedlings probably would have been naturally counterselected due to the expression of deleterious genes. Unfortunately, this short-term advantage is also a long-term dead end as there is no more possibility for genetic evolution.

Production of haploid genotypes: Our findings also demonstrate the opportunity to produce haploid genotypes in *C. sempervirens* species. This material is highly valuable for genetic studies and mapping, especially in *C. sempervirens* where the tetrasporic origin of the megagametophyte (EL MAËTAOUI *et al.* 1998) prevents analysis of marker segregation as usually performed in conifers (WU *et al.* 1999). Finally, the highly probable sterility of the haploid genotypes offers a unique opportunity of producing cone-free cultivars. This may be very attractive in practical terms since the heavy production of female cones depreciates the aesthetic value of ornamental cypresses, while pollen produced by male cones causes severe pollinosis in Mediterranean regions (CHARPIN *et al.* 2005).

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