Summary. Plants were regenerated from axenic plantlets by mesophyll protoplast culture, without mutagenic treatment. Two different lines of *Nicotiana sylvestris* were used: an original line, and a diploid androgenetic line derived from it. The regenerated plants were either diploid and phenotypically similar to their respective protoplast source line, or tetraploid. Genetic studies carried out on several diploid regenerated plants revealed genetic variability. Eight of 13 selfed progenies of plants regenerated from the original line, and 1 of 8 selfed progenies of plants regenerated from the androgenetic line, produced new mutant phenotypes never observed in the protoplast source lines. Two plants regenerated from the same protoplast-derived callus produced different mutations. Selfed progenies without a recognizable mutant phenotype were also different from their respective protoplast source line for quantitative characters; protoplast culture induced a depressive effect on the size of plants derived from protoplasts at younger and older stages of development. The origin of this depression and of the mutations is discussed.

Key words: Induced genetic variability – Genetic studies – *Nicotiana sylvestris* – Protoplast culture

Introduction

Several authors have pointed out the great usefulness of protoplast culture in plant breeding (Bhojwani et al. 1977; Vasil et al. 1979; Shepard 1982): in multiplying plants of various species (Wenzel et al. 1979), in obtaining drug resistance mutants and auxotrophs (Carlson 1970; Bourgin 1978; Maliga 1980), and in somatic hybridization (Belliard and Pelletier 1978; Zelcer et al. 1978; White and Vasil 1979). However these authors did not study the genetics of plants regenerated in the absence of mutagenic treatment or somatic hybridization.

*Nicotiana sylvestris* was chosen for this study because: (1) its protoplast culture has been perfected (Nagy and Maliga 1976; Bourgin et al. 1976; Durand 1979; Magnien et al. 1980; Negruțiu and Mousseau 1980), (2) the diploid level (2n=24) of this species facilitates genetic study of regenerated plants, and (3) the phenotypic stability (Goodspeed and Avery 1939) in successive selfed progenies allows one to think that any new phenotypes observed would be induced by protoplast culture. Therefore, the aim of this paper is the analysis of genetic variability found in plants regenerated by mesophyll protoplast culture. Two diploid protoplast source lines were used (Fig. 1): one was the original line, maintained by successive self-pollinations, the other was a doubled haploid line, obtained from the original line after several consecutive cycles of androgenesis (De Paepe et al. 1981). The androgenetic line, theoretically homozygote, was phenotypically different from the original line ("crumpled" phenotype and reduced vigour) as are most lines obtained by androgenesis in *Nicotiana sylvestris* (De Paepe et al. 1981; Prat 1982). We thought it would be interesting to compare the induced variability in protoplast culture of an androgenetic line to that of the original line. Morphological and quantitative characters of diploid plants regenerated from both protoplast source lines were analysed genetically.

Material and Methods

Protoplast Source Lines

The original diploid line of *Nicotiana sylvestris*, supplied by the SEITA of Bergerac, has been maintained by 7 successive self-pollinations in greenhouses at Gif-sur-Yvette. No phenotypic variability was observed in successive selfed progenies.

The androgenetic line was diploid and obtained from the original line by 5 consecutive cycles of pollen culture (De
overnight enzymatic incubation in the dark at 19 °C, the flasks were gently shaken. The protoplast suspension was poured through nylon sieves (150 μm aperture), and centrifuged 7 min at 800 rpm (about 70 g). The protoplast pellet was resuspended in culture medium (Durand 1979) in which we substitute glutamine and asparagine for the conditioned medium, and sorbitol for mannitol. After determination of the protoplast density on a Nageotte haemocytometer, the protoplast suspension was adjusted to 25,000 living (Durand 1979) protoplasts per milliliter and poured into Petri dishes (1.5 ml per dish, 5 cm in diameter) sealed with Parafilm, kept at 23 °C in the dark for one week and then at 25 °C under low light intensity (1,500 lux). Protoplasts developed into calli that were planted out in Petri dishes on solid medium (Bourgin et al. 1979) inducing shoot formation. The shoots were planted out on B medium of Bourgin et al. (1979) induced rooting. Afterwards plantlets were planted in the greenhouse, about 5–6 months after sowing the protoplast source plants.

**Chromosome Counts**

Mitotic counts were carried out on root tip squashes stained by the Feulgen reaction. Root tips were taken from young plants, immediately treated by 2 mM hydroxyquinoline for 4 h at 8 °C and then fixed in acetic alcohol (1:3 v/v). Root tips were hydrolysed in 5 N HCl for 10 min, and then washed in water and stained by Schiff’s reagent for 30 min. The chromosome counts were checked at meiosis. Flower buds were taken and fixed in Carnoy’s solution (alcohol, chloroform, acetic acid; 6:3:1; v/v/v), and anthers were stained in acetocarmine.

**Genetic Studies**

**Morphology.** Regenerated plants were compared with their respective protoplast source lines. Selfed progenies of several diploid regenerated plants were also examined. When a previously unknown phenotype was observed in the progenies, self-pollinations, crosses between the new phenotype and its protoplast source line, and then self-pollinations of the obtained hybrids were carried out to study the heredity of the obtained mutations.

**Quantitative Genetics.** Only diploid regenerated plants were affected by this study. A comparative quantitative test was carried out: some selfed progenies of protoplast-regenerated plants taken at random from each protoplast source line were compared to progenies of plants obtained by selfing the respective protoplast source line, for several characters i.e.: leaf size at different stages of development (rosette plant, after induction of flowering, flowering plant, plant bearing mature seeds), height, and flower size. All compared plants were sown the same day (in culture tubes, on Knop’s medium). Forty day-old seedlings were replanted in the greenhouse (photoperiod: 16 h; thermoperiod: 24 °C/17 °C), watered with the nutritive solution of the Phytotron (Nitsch 1969), and then measured the same day or at the same stage of development. Plants were distributed into randomised blocks with the same number of plants for each progeny.

**Statistical Tests**

Segregations of mutant phenotypes were tested with chi square (Dagnelie 1980). Comparisons of quantitative characters were tested by the contrast method (Dagnelie 1980): the means of selfed progenies of plants regenerated from each

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Fig. 1. Protoplast source lines. Original line (left) and androgenetic line (right) with crumpled leaves