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Shoot regeneration in long-term callus cultures derived from mature flowering plants of *Cyclamen persicum* Mill.

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Summary. In long-term callus cultures of *Cyclamen persicum* Mill. two types of tissue could be distinguished. One type featured a brown suberised outer layer and was poorly organogenic. The other type was yellowish in appearance and gave rise to many shoot buds. Both types co-existed on the same callus, the former prevailing. Selection for organogenic tissue resulted in cultures yielding approximately three times more petioles than random subcultures. Callus-derived shoots could be rooted and established in the greenhouse. The method allowed for the production of thousands of plants but the regenerants often showed deviant phenotypes and genotypes.

Abbreviations: BA, 6-benzylaminopurine; BMP, basal medium propagation; BMR, basal medium rooting; DAPI, 4',6-diamino-2-phenylindole; KIBA, potassium salt of indole-3-butyric acid; kinetin, 6-furfurylaminopurine; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid

Key words: *Primulaceae*, tissue culture, regeneration, somaclonal variation, organogenesis

Introduction

In vitro regeneration of *Cyclamen persicum* Mill. has been attempted in various ways (reviewed by Geier et al. 1990). Regeneration from seedling tissue has met with some success but is of little significance for breeding purposes. Unfortunately, when compared to seedling tissue, mature flowering plants exhibit a reduced competence for organogenesis.

Recent reports have focused on direct regeneration of shoots from leaves, petioles and peduncles of flowering plants (Murasaki and Tsurushima 1988, Dohm et al. 1991, Schwenkel and Grunewaldt 1991). On the other hand, regeneration from callus derived from mature tissues has rarely been achieved. Loewenberg (1969) reported that shoots developed sporadically on tuber-derived callus but no viable plants could be established from these cultures. Geier (1977) found that anther-derived callus cultures gradually acquired and retained organogenic competence. However, shoot buds appeared only occasionally and generally did not survive hardening. Similar results were obtained with leaf-derived cultures.

In this report we describe efficient regeneration from long-term callus cultures that were derived from explants of flowering plants. The potential of the method for application in micropropagation is assessed.

Materials and methods

Tissue culture. Cyclamen persicum Mill. plants were grown in a greenhouse until flowering. Young leaves of approximately 45 mm length and 60 mm width were dipped in 70% (v\v) ethanol. After 15 min in 1.5% NaOCl (added as 'Glorix', a commercial solution containing detergent), leaves were rinsed four times in sterile water. Leaf discs of 1 cm^2 were excised and placed on the medium abaxial side down. The basal medium for propagation (BMP) consisted of MS salts and organic addenda (Murashige and Skoog 1962), 8 $g \cdot l^{-1}$ agar (Merck 1614), 60 $g \cdot l^{-1}$ sucrose and BA, kinetin and NAA at various concentrations.

The basal medium for rooting (BMR) consisted of Nitsch salts and organic addenda (Nitsch 1969), 8 $g \cdot l^{-1}$ agar (Merck 1614), 60 $g \cdot l^{-1}$ sucrose and 1.0 $mg \cdot l^{-1}$ KIBA. Medium pH was adjusted to 5.8 at room temperature before addition of agar. Medium was dispensed into glass jars with polypropylene screw-on lids and autoclaved for 20 minutes at 121°C.

For regeneration experiments treatments consisted of at least 30 callus pieces. For rooting experiments treatments contained on average 30 shoots.

All cultures were kept at 18 ± 1°C in complete darkness (initiation, propagation and shoot regeneration) or at 5000 to 6000 lux (rooting).

Flowcytometric analysis. 1-cm^2 leaf pieces of regenerants were chopped in 2 ml Ficoll buffer (Dunn and Wobbe 1993) and centrifuged for 2 min at 700 g. After removal of the supernatant, the pellet was resuspended in 1 ml of commercial buffer (Partec) containing DAPI and sieved over a 30-μm nylon sieve. Analyses were carried out using a Partec CA-II flow cytometer.

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Results

Shoot regeneration

Seventeen weeks after initiation of leaf disc cultures (line G-2) on the BMP with 2.0 mg·l^{-1} NAA and 3.2 to 4.0 mg·l^{-1} BA and kinetin, abundant callus formation was observed. Callus was excised from the original explants, cut into pieces of roughly 1 cm^3 and subcultured on the same medium. After three subcultures lasting nine, eight and ten weeks, callus was subcultured a further five times on the BMP with 3.0 mg·l^{-1} NAA and 4.0 mg·l^{-1} BA and kinetin. Each subculture lasted six to seven weeks.

The initial callus was white and unorganised. After a few subcultures the majority of the callus featured a typically brown suberised outer layer. Little or no organogenesis was observed on this type of callus. However, on part of the callus pieces patches of tissue of a totally different morphology had appeared. This tissue was yellowish, only slightly suberised and highly organogenic. The organogenic sectors contained numerous meristems that developed into leaves towards the end of each subculture. Since propagation cultures were kept in the dark, these leaves featured etiolated petioles and ill-developed laminae. After removal of leaves, both organogenic and non-organogenic tissue was selected and transferred to fresh medium (Fig.1). The organogenic competence of the different tissue types was assessed by counting the number of etiolated petioles 6 weeks after transfer. As a control, callus was also randomly subcultured.

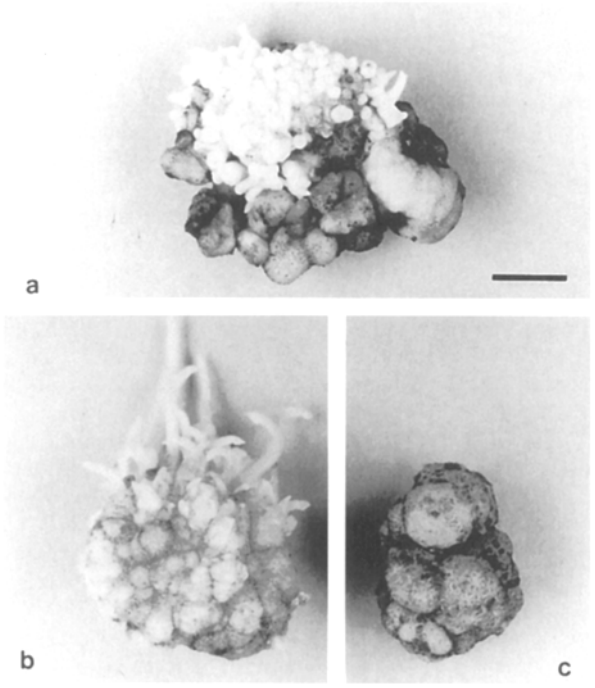

Fig.1. (a) Callus featuring organogenic and non-organogenic sectors, (b) selected organogenic callus, and (c) selected non-organogenic callus. Bar=5 mm.

Non-organogenic callus yielded non-organogenic callus with few exceptions. Organogenic tissue yielded more organogenic tissue. As a result, compared to control cultures, average petiole yield per callus piece was three times higher in cultures from selected organogenic tissue (Fig.2). The selected tissue lines retained their organogenic competence in the course of subsequent subcultures for at least two years.

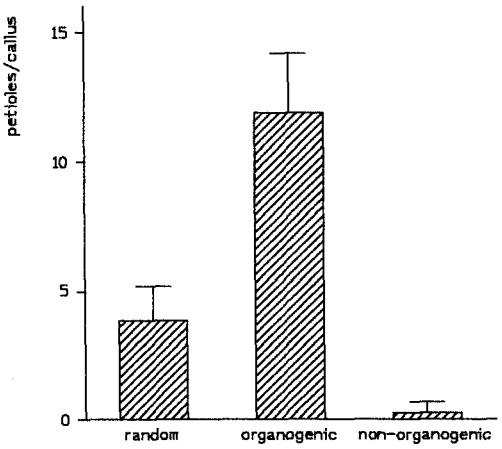

Fig.2. Petiole yield of randomly subcultured, selected organogenic and selected non-organogenic callus, 6 weeks after transfer. Error bars indicate 95% confidence intervals. Analysis of variance revealed a significant effect of the different treatments at the 1% confidence level.

The procedure of callus selection could be successfully applied to different genotypes cultured under various growth regulator conditions.

Rooting and hardening of shoots

Five to six weeks after transfer to fresh medium, organogenic callus contained numerous etiolated shoots (Fig.3). These shoots consisted of one or more leaves, bearing one or more petioles at their base. Excised shoots could be rooted in the light.

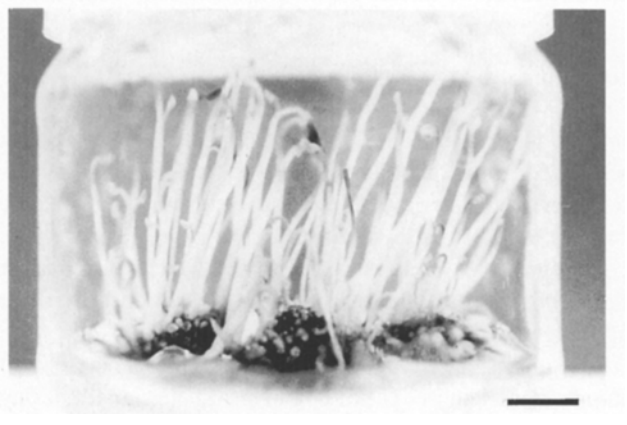

Fig.3. Etiolated shoots on 6-week old organogenic callus cultures. Bar=12 mm.

Rooting was more efficient on medium (BMR) containing Nitsch macro salts (Nitsch 1969) than on the same medium containing MS (Murashige and Skoog 1962) macro salts. In a typical experiment, 88% of the excised shoots rooted on BMR whereas with MS macro salts only 53% rooting was achieved.

A relatively high concentration of sucrose was a prerequisite for efficient rooting. At 30 $g \cdot l^{-1}$ no rooting was observed, whereas at 60 $g \cdot l^{-1}$ rooting was almost complete after four weeks (Fig.4).

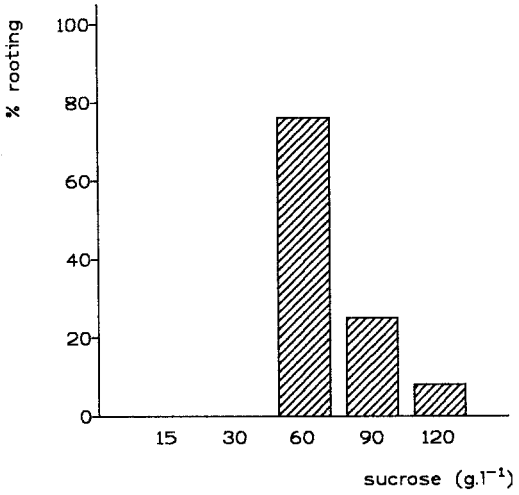

Fig.4. The effect of sucrose concentration on rooting.

Three main shoot types could be distinguished on organogenic callus. A 'shoot' consisted either of a single meristem with a petiole attached to it (type 1, Fig.5A) or of a single meristem in between two petioles (type 2, Fig.5B), or of a cluster of several petioles and meristems (type 3, Fig.5C).

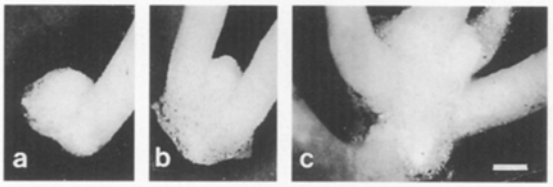

Fig.5. Bases of shoots consisting of: (a) one leaf with one meristem, (b) a meristem in between two leaves, and (c) a cluster of several leaves and meristems. Bar=1 mm.

The different shoot types could be rooted with equal success but behaved differently when transplanted to the soil. In a typical experiment, 38, 70 and 92% of type 1, type 2 and type 3 shoots respectively survived the transfer to the greenhouse. It was observed that type 1 shoots, and to a lesser extent type 2 shoots, developed few new leaves during acclimatization. Instead, pre-existing leaves strongly expanded. On the other hand, type 3 shoots rapidly developed new leaves *ex vitrum*.

The above described rooting conditions were established for one genotype (line E-8). We tested the applicability of the rooting protocol on 1200 shoots of 18 genotypes belonging to 7 inbred lines (it should be noted that *Cyclamen* can only be selfed to a limited extent and therefore 'inbred' lines are heterogeneous). The different genotypes were propagated under different growth regulator conditions with selection for organogenic callus as described above. On average, 90% of the shoots could be rooted and 78% could be established in the soil (Fig.6).

Fig.6. Overview of regenerants in the greenhouse.

Stability

After 2 years of callus propagation, 2400 plants regenerated from 21 genotypes belonging to 8 inbred lines were grown to maturity in a greenhouse. Plants were visually inspected for phenotypic aberrations.

A significant fraction of the population featured thick succulent leaves, swollen petioles and peduncles and a reduced number of leaves and flowers. Ploidy determinations on randomly selected plants of this type always revealed an increased (and at least doubled) ploidy level. On the other hand, no ploidy alterations could be detected in plants that looked normal. Of 21 genotypes, 11 yielded regenerants with this deviant phenotype. In one line, of which 3 genotypes were tested, all regenerants were aberrant. In 3 lines (1,2 and 2 genotypes tested) each genotype yielded 10 to 20% aberrant regenerants. In one line, 3 out of 8 genotypes tested each contained 10 to 20% deviant plants whereas in the other genotypes of this line all plants looked normal. In 3 lines (1,1 and 3 genotypes tested) no aberrant plants were encountered. It therefore seems that stability was genetically determined.

Discussion

Long-term callus cultures of *Cyclamen* featured morphologically distinct tissue types. Selection for either type yielded callus lines that largely retained the characteristics of the tissue from which they had been derived. The occurrence

of different callus types in long-term cultures has been reported for other species such as *Triticum aestivum* (Redway et al. 1990), *Zea mays* (Lu et al. 1982), *Zea diploperennis* (Swedlund and Locy 1988), *Saccharum* spp. (Fitch and Moore 1990) and *Panicum miliaceum* (Heyser and Nabors 1982). In these systems, the feasibility of selection for certain tissue types to improve regeneration success has been demonstrated. It is clear that the same strategy can be applied to *Cyclamen* callus cultures. The underlying mechanisms governing the occurrence of different tissue types in cultures derived from the same explant remain to be investigated.

Wicart et al. (1984), working with one genotype of *Cyclamen*, observed shoot buds, roots, unipolar tubers, bipolar tubers and somatic embryos on the same callus. Throughout numerous subcultures under different growth regulator conditions, we never observed bipolar structures. Without exception, regeneration took place through organogenesis.

The described procedure enabled the production of thousands of micropropagated plants. This rate of success is comparable to that obtained with direct regeneration from peduncle explants (Schwenkel and Grunewaldt 1991). Since cultures could be initiated from mature flowering plants, the method has potential for application in breeding programs. However, we found that initiation of organogenic callus cultures was poorly reproducible. For example, organogenic cultures of a certain line could be established in one year but not in the next year. Moreover, the stability of long-term callus cultures can be questioned. Morphological aberrations were observed, particularly after prolonged callus propagation. Deviant plants featured succulent leaves and reduced branching, correlating with an increased ploidy level. Less dramatic genetic alterations that did not result in an altered phenotype may have occurred. The frequency of instability was genotype-dependent as was also observed for plants produced through direct regeneration (Schwenkel and Grunewaldt 1991). Analysis of 6000 plants of 8 cultivars produced in this way revealed that, as in our system, ploidy alteration was the major type of aberration. It occurred in all tested cultivars. In addition, regenerants of some cultivars featured flower deformations. We almost never observed this type of aberration. This discrepancy may relate to the regeneration technique used, i.e. direct shoot formation from peduncle explants (Schwenkel and Grunewaldt 1991) as opposed to regeneration from long-term callus cultures (this work). On the other hand, it should be noted that the donor plant material used in the two systems differed. In this study, inbred material was used whereas Schwenkel and Grunewaldt (1991) employed F1-hybrid or open-pollinated cultivars. Phenotypic aberrations were also observed in plants propagated through somatic embryogenesis and increased with increasing cycle number (Kiviharju et al. 1992).

The repeatedly encountered instability of *Cyclamen* tissue cultures jeopardises the application of *in vitro* regeneration of this species for breeding and other purposes.

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