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Meetings of the Royal Botanical Society of The Netherlands

MEETING OF THE SECTION FOR PLANT SYSTEMATICS AND GEOGRAPHY ON 24 APRIL 1992

Significance of Cytological Characters in Systematic and Evolutionary Studies of Crassulaceae

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Cytologically the family Crassulaceae is probably the most diverse group of angiosperms. Chromosome numbers range from $2n=8$ to $2n=640$, and basic numbers from $x=4$ to $x=37$ and more. The genus *Sedum* especially is extremely variable. The 54 European species for instance, comprise about 140 different cytotypes.

Chromosomes of Crassulaceae are generally very small, usually less than 2 μm, but variation in chromosome size is nevertheless considerable. Chromosome length may vary 10-fold among species as well as among chromosomes of a single karyotype. The amount of DNA per nucleus is proportional to chromosome number and chromosome length. Although 2C values are extremely low in many species (0·5 pg and less), 40-fold differences have been observed among species.

Cytological variation in *Sedum* is due to autopolyploidy, descending dysploidy (Robertsonian translocations), and amphiploidy, and in most cases the direction of the transformations of the cytological characters can be determined unequivocally. Furthermore, variation in 2C values is often correlated with life-form or adaptations to more extreme habitats. In small infrageneric groups, cytological variation is often a powerful tool for reconstructing phylogenies. For instance, in *Sedum* series *Rupestria* the phylogeny, based on cytological variation, fully agrees with a cladogram based on 240 different characters states of 104 morphological characters.

Among the European infrageneric taxa of *Sedum*, different levels of chromosomal evolution can be distinguished. Groups either have a single basic chromosome number or their basic number varies. Some groups have low primary basic numbers ranging from $x=5$ to $x=13$, whereas others comprise primary as well as secondary basic numbers (from $x=6$ to $x=37$), or have only secondary ($x=14$ or higher) basic numbers. A similar pattern of cytological variation can be observed among genera and subfamilies. However, the evolutionary significance of the cytological variation among these higher taxa is not yet clear.

Chloroplast DNA Variation and Evolutionary Relationships in the Crassulaceae

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Evolutionary relationships within the Crassulaceae were investigated at various taxonomic levels. To investigate characters independent from those thus far used ('t Hart, H. (1991): *Flora Mediterranea* **1:** 31–61) we studied variation in the conservatively evolving chloroplast genome by comparative restriction-site mapping. As a basis for this study we generated a complete clone bank of the chloroplast DNA (cpDNA) of the European *Sedum album* L. from which clones were used as homologous probes in species comparisons (van Ham, R.C.H.J. *et al.* 1992. *Biochem. Syst. Ecol.* **20:** 243–253).

CpDNA restriction-site variation in *Sedum* series *Rupestria* revealed new insights in the evolution of the seven species of this comparium, i.e. a different basal branching and a more complicated reticulate terminal branching pattern including one taxon (*S. montanum* ssp. *montanum*) of putative triple hybrid origin. A preliminary analysis of restriction-site variation at the familial level provided evidence for a basal split in the family between a *Crassula*-lineage and a *Sedum*-lineage. Within the *Crassula*-lineage, the divergence between *Crassula* and the disputed genus *Tillaea* must have taken place relatively early in the evolutionary history of the family. The evolution of the *Sedum*-lineage is much more diverse and complicated. The analyses suggest that (a) the classical subfamilies Kalanchoideae, Cotyledonoideae, Sempervivoideae and Echeverioideae are terminal taxa of the *Sedum*-lineage; (b) there is a close, possibly monophyletic, relationship between the Kalanchoideae and Cotyledonoideae; (c) the genus *Sedum*, is paraphyletic and possibly comprises three major lineages, each of which has members on more than one continent. Finally, a parsimony analysis of morphological characters showed little congruence with the cpDNA phylogeny, indicating that parallelism greatly contributed to the phenotypic diversification in the Crassulaceae.

Rosularia (Crassulaceae)—A Systematic Study

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Rosularia (De Candolle) Stapf 1923, based on *Umbilicus* sect. *Rosularia* De Candolle 1828, was monographed by the author (Eggli, U. (1988): *Bradleya* **6:** (Suppl.) 1–120) and counts 36 taxa (26 species including one described after the completion of the monograph and 10 infraspecific taxa).

The genus is closely related to *Sedum* in most characters and is accordingly placed in subfamily *Sedoideae*, at variance with Bergers' treatment. It differs from *Sedum*, in that its leaves are always rosulate, its petals are sympetaly (petals united for one-tenth to three-quarters of their length), and its carpels remain erect even at fruiting time. The pollen is tricolporate and may be without ornamentation, or rugulate-striate; the seeds are of the bipapillate type and are longitudinally striate to costate. Cytologically, the genus falls into two widely dissimilar groups, either with $x=7$ or $x=9$.

The genus is classified into four sections on the base of comparative morphology and cytology. Section *Rosularia* is widespread in the Near and Middle East (from Crete to Afghanistan), has $x=9$, and the only polyploids known are tetraploids which are characteristic for three Turkish subspecies of the widespread *R. sempervivum*.

Section *Sempervivella* (= *Sempervivella* as separate genus; but also including *Sedum hirsutum*) is widely distributed in the western Mediterranean, North Africa, Ethiopia, and the Hindukush–Himalaya region; its largely disjunct area is interpreted as being relictic. The section is cytologically heterogeneous, but at least some taxa also have $x=9$.

Section *Ornithogalopsis* is distributed in the Himalaya–Hindukush area and in South Inner Asia. This is the least well-known section; the basic chromosome number seems to be $x=7$, and all specimens investigated were tetraploid.

Section *Chrysanthae* has a relatively narrow distribution in the mountains of Turkey and immediately adjacent regions of Iran, Iraq and Soviet Armenia. The base number is $x=7$, with predominant polyploidy (up to 16 ×) in all taxa investigated; all taxa are interpreted as neoendemics.

Alkaloids of some European Sedoideae and Sempervivoideae

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Of the Crassulaceae investigated for the presence of alkaloids, *Sedum acre* has received most attention. Some 20 piperidine alkaloids have been reported for this species alone (Hegnauer, R. (1989): *Chemotaxononie der Planzen*, **8**, Birkhäuser, Basel). *S. acre* has retained a combination of morphological characters, which are considered to be primitive within the Crassulaceae ('t Hart, H. & Koek-Noorman, J. (1989): *Taxon* **38:** 535). Because a similar combination of primitive characters occurrs in *S. aetnense, S. anglicum, S. brissemoreti, S. farinosum, S. fusiforme, S. lancerottense, S. melanantherum*, and *S. nudum*, we investigated these species for the presence of alkaloids. A number of pyrrolidine and piperidine alkaloids, some of which have not been reported as natural constituents, were detected in the leafy parts.

The distribution of the alkaloids agrees perfectly well with the infrageneric classification of the European and Macaronesian *Sedum* species ('t Hart, H. (1991): *Flora Mediterranea* **1:** 31), except for *S. farinosum* of *S.* series *Macaronesia. S. farinosum* should be separated from the other species of *S.* series *Macaronesia*, i.e. *S. brissemoreti, S. fusiforme, S. lancerottense*, and *S. nudum*, based on its alkaloids composition, ecological preference, polyploidy level, colour of the flowers, and its hybridization pattern.

Primitive as well as advanced morphological characters are present in the *acre*-group, which comprises *S. ursi, S. alpestre, S. grisebachii, S. laconicum, S. annuum, S. borissovae, S. tuberiferum, S. urvillei, S. apoleipon, S. multiceps, S. sexangulare, S. samium*, and *S. litoreum* in addition to *S. acre* itself. Except for *S. acre*, these species were found to contain only pyrrolidines and 2-monosubstituted piperidines of the pelletierine-type. A good concordance was observed between the composition of alkaloids and the hybridization pattern.

In contrast to the presence of alkaloids in the more primitive *Sedum* species, pyrrolidine and piperidine alkaloids were absent in species of *Sedum* series *Rupestria, Aeonium, Greenovia, Jovibarba*, and *Sempervivum*, which all share the same combination of advanced characters. These results indicate a correlation between the distribution of alkaloids and the major evolutionary trends in the European Crassulaceae. Whether the occurrence of alkaloids may be considered a primitive character in other groups of Sedoideae as well is not yet quite clear.

Systematics of *Monanthes* (Crassulaceae)

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Monanthes is a small genus of 10 species and one heterotypic subspecies. It is mainly characterized by its enlarged, petaloid nectaries. Furthermore *Monanthes*

is remarkable for its partly extensive development of bladder-cell idioblasts. Except for two species (one occurs in the Atlas Mountains of Morocco, and one is restricted to a few localities on the Salvage Islands), all taxa are confined to the Canary Islands. The most useful characters for the distinction of the recognized taxa proved to be growth form, origin of inflorescence (vegetative axes determinate or indeterminate), petal shape, nectary shape, and the size and distribution of bladder-cells and glandular hairs. Based on their growth form and supported by several other characters, the perennial members of *Monanthes* are easily assigned to one of three sections. Distribution patterns and ecological preferences support this classification. Section *Monanthes* includes *M. polyphylla* ssp. *polyphylla* and *M. polyphylla* ssp. *amydros*, *M. muralis*; Section *Petrophyllea* includes *M. atlantica*, *M. lowei*, *M. brachycaulos*, *M. minima*, *M. pallens* and *M. icterica* (annual; included here for its floral morphology); Section *Sedoidea* includes *M. anagensis* and *M. laxiflora*. the morphological diversity found in this small genus facilitates speculations on possible pathways of the evolution. Several, especially floral characters support the assumption that the members of Section *Monanthes* and Section *Sedoidea* are advanced relative to those of Section *Petrophyllea* (which includes character states generally referred to groups thought to be rather primitive in the Crassulaceae). The same data makes obvious that the two derived groups have different origins within Section *Petrophyllea*. *M. atlantica* (often treated under *Sedum* (*S. surculosum*) most probably relates *Monanthes* with taxa actually referred to *Rosularia* (especially *R. jaccardiana*).

Evolutionary Relationships in *Aeonium* (Crassulaceae)

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The genus *Aeonium* is largely confined to the Canary Islands. Thirty-two of the 37 species and subspecies occur on the Canary Islands; two species occur on Madeira, one on the Cape Verde Islands and Morocco and two are found in East Africa and Arabia. *Aeonium* is generally considered to be a young genus in which the variety of growth forms is a result of adaptive radiation. Restriction patterns of chloroplast DNA (cpDNA) were used to study evolutionary relationships among 12 *Aeonium* species. CpDNA was digested with 12 restriction enzymes recognizing six base-pair sites. Divergence of cpDNA in *Aeonium* is very low. The fraction (F) of shared fragments (fragments of identical size) varies between 0·92 and 0·99. Remarkably, three of the geographically most separated species appear to be especially closely related: *A. gorgoneum* (Cape Verde Islands), *A. holochrysum* (Canary Islands) and *A. leucoblepharum* (Yemen). They have almost identical RFLP patterns. CpDNA-divergence among these species is comparable to that of two populations of *A. spathulatum* from different islands. It can be concluded that the aforementioned species have diverged recently and certainly do not represent the ancestral forms in *Aeonium* as suggested by Lems (Lems, K. (1960): *Ecology* **41**: 1–17). Lems came to this conclusion on the basis of growth form and geographical distribution. Our results are also in conflict with Liu (Liu, H.-Y. (1989): *Nat. Mus. Nat. Sci.* **1**) who even classified the three species in two different sections.

CpDNA of *Greenovia aurea* does not differ significantly from that of *Aeonium*. The F values vary between 0·92 and 0·96 and are comparable to the F values of cpDNA within *Aeonium*. These cpDNA data suggest that *Greenovia* should be included in *Aeonium*.

Variation and Evolution of Crassulacean Acid Metabolism in *Sedum* and *Aeonium*

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Crassulacean acid metabolism is a photosynthetic pathway which can be considered as an adaptation to arid environments. The CAM pathway has evolved from the 'normal' C3 pathway, and has developed independently in many plant families. The main subject of our investigations was the evolution of CAM in *Sedum* and *Aeonium*. Using several parameters, the variation of CAM within these genera was analysed and evolutionary models are proposed.

Analysis of the variation of CAM in *Sedum* showed a distinct difference between the Mexican (obligate CAM) and the European (facultative CAM) species. The variation in CAM and *Sedum* agreed to some extent with the major evolutionary trends within the genus. Species from the more advanced groups generally had a higher CAM activity than less specialized species, even though certain members of the latter group have developed a high CAM activity in the dry climate of Southern Madeira. Consequently, CAM may have evolved more than once in this genus and we assume that all *Sedum* taxa have the ability to develop CAM.

The large variability of CAM in *Aeonium* appeared to be correlated with the habitat of the species (strong CAM in arid habitats), as well as with the growth form and taxonomic classification into sections. From these results we conclude that CAM probably evolved in the monophyletic genus *Aeonium* parallel with the morphological differentiation, from small undifferentiated *Sedum*-like species, displaying little or no CAM, via

rosettes and/or candelabrum-shaped forms with intermediate or strong CAM, to large shrubby species displaying strong CAM activity. As a possible scenario we propose the following succession of events. *Aeonium* evolved from an *Aichryson*-like ancestor, which in turn was derived from a *Sedum*-like progenitor, probably resembling the present-day *S. gattefossei* from Morocco. The diversity of present-day species rapidly evolved from this ancestor through adaptive radiation (comparable to Darwin's finches), with similar species occupying comparable niches on different islands.

Genetic Variation and Interspecific Hybridization in *Kalanchoë*

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The genus *Kalanchoë* Adanson ($2n = 34, 68, 102, 136$) is divided in the sections *Kitchingia, Bryophyllum* and *Kalanchoë*. It contains *c.* 150 species, 125 of which are (cyto)taxonomically determined (Baldwin, J.T. (1938): *Am. J. Bot.* **25:** 572–579; Hamet, R. & Marnier-Lapostolle, J. (1964): *Arch. Mus. Hist. Nat.* (Ser. 7) **8:** 1–110). Species of the sections *Kitchingia* and *Bryophyllum* have drooping flowers, plants of the section *Kalanchoë* have upright flowers. The genus exhibits considerable genetic variation. This variation should enable plant breeders to obtain a further improved assortment for both pot plants and cut flowers. For evaluation of important characters of wild species, 116 species and 47 F1-hybrids were studied. The most important attributes measured were: mean reaction time (number of days from the beginning of short-day treatment to flowering), stem length and endurance of individual flowers. Minimum reaction time was found in *K. rotundifolia* ssp. *strictifolia* (58 days). Stem length varied from 5 cm in *K. guignardii* to 95 cm in *K. velutina*. Endurance of flowers varied from 4 days in *K. afzeliana, K. densiflora, K. gracilipes, K. lateritia* and *K. serrata* to 36 days in *K. blossfeldiana*. Ten species were selected for interspecific diallelic hybridization. Only a limited number of hybrids was obtained. According to our results crossing barriers are not related with the current systematic classification of the genus but seem to depend on specific combinations of species used. Nature and location of crossing barriers have been studied. Promising hybrids, partly with restored fertility will be released to commercial plant breeders.

MEETING OF THE SECTION FOR FERTILIZATION RESEARCH IN PLANTS ON 21 FEBRUARY 1992

Cytological Analysis of Apomixis and Sexuality in *Poa pratensis* L.

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Interest in apomixis has recently increased in breeding, biotechnological and genetic engineering programs. Therefore it is imperative that time-saving and informative techniques are developed for screening wild and cultivated species for apomixis as well as for determining the degree of apomixis and estimating ploidy levels of endosperm and embryo cells. Classical cytological techniques are not really useful for investigating numerous ovules and embryo sacs or for estimating ploidy levels. Methods for complete embryo-sac investigation have been developed in Russia. These techniques enable the study of numerous embryo sacs, the identification of polyembryony, the cytological analysis of the endosperm and the estimation of embryo-sac fertility.

Twelve *Poa pratensis* genotypes were investigated after self- and open-pollination and reciprocal crosses and included in a research programme of CPRO-DLO. Some genotypes showed a tendency to apomixis as estimated from homogeneity in their offspring. Material was collected from greenhouse grown plants, cultured at 18°C, 2–11 days after pollination, and fixed in FAA and stored in ethanol 70%.

Ovules were dissected from the ovaries, embryo sacs were taken out from the ovules by hand with fine needles under binocular microscopes. Embryo sacs were then stained for 10 min with the DNA-specific fluorochrome (DAPI) (10^{-5} M DAPI buffered (pH = 4·0) solution), embedded in glycerin–gelatin and examined and photographed under phase-contrast and interference contrast microscopes. A microcytophotometer with a Phloem incident UV light with an excitation wave length of 365 nm was used to measure the amount of DNA in the intact nuclei of endosperm, embryo and diploid nucellus cells. Excitation wave length was 365 nm, emission wave length was 470 nm. Measurements were taken from at least 10 different areas of 16×16 µm at a magnification of $\times 240$.

The analysis of complete ovules and embryo sacs indicated that most of the *Poa pratensis* genotypes studied, display a tendency to apomictic seed reproduction but there are differences in degree of amphimixis and apomixis: (a) predominant apomictic seed development; (b) apomictic and amphimictic reproduction more or less in balance; (c) amphimixis predominant. Viable seeds in *P. pratensis* result for

amphimixis after double fertilization as well as from parthenogenetic embryo development and endosperm formation after single fertilization. Embryo development without endosperm is necessarily the result of diploid parthenogenesis. These seeds are not viable in nature but abort. The failure of endosperm development in the absence of fertilization is the main reason for seed degeneration in case of apospory and parthenogenesis. A tendency to normal (1*x* genome) and aposporic (2*x*) embryo-sac formation in the same plant was observed in many *P. pratensis* genotypes and confirmed by the cytophotometric measurements, which gave evidence of 3*x* and 5*x* endosperms. This technique could give quantitative data on the prevalence of apo- and amphimixis in individual genotypes in a breeding programme.

Attempts to find the Genetic Back-Ground of Apomixis in *Poa pratensis* L.

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Poa pratensis L. (Kentucky bluegrass) is one of the most important temperate grasses used both in pastures, for fodder and for lawns. Most cultivars are highly polyploid aneuploids ($2n = 36$–82, with $x = 7$) which reproduce mainly via aposporous apomixis. This method of asexual seed production (agamospory) maintains the genotype of the maternal parent despite the fact that pollination and fertilization of the endosperm is required for seed development (pseudogamy). Plant breeders, however, face difficulties in generating and exploiting genetic variation for the selection of new improved cultivars.

Although many accessions of the species are facultative apomictic (i.e. reproduce both sexually and via apomixis, depending on genotype and environment), selection of cultivars has strongly favoured a very high degree of apomixis. Crosses with sexual types generally lead to loss of apomixis, which is only very erratically recovered. The genetic basis of apomixis in *Poa pratensis* is not known, whereas in some other species a relatively simple hereditary basis is postulated, one reason for this being the high chromosome numbers of available genotypes. CPRO-DLO started a project several years ago to obtain contrasting genotypes with a lower ploidy level to be crossed for genetic analysis. Twin seedlings were extracted; these occur in relatively high frequency in this and other facultative apomictic species. Twin percentages of up to 8% were found. About one-tenth of these twins were visibly non-identical. Of these the less vigorous halves were evaluated, as these individuals may have developed from a non-fertilized, sexual, numerically reduced embryo-sac (haploid parthenogenesis). Chromosome numbers were estimated via flow cytometry (FCM) and apomictic tendency via progeny testing. The majority of the plants contained chromosome numbers as high as or higher than the parent, but several plants with reduced chromosome numbers were selected. Some of these reproduced (mainly) via apomixis as evidenced by the homogeneity of their progeny. As their ploidy was considered still too high, the twin-seedling extraction procedure was repeated. Twin percentages of up to 20% were observed, and there was no relation between twin percentage of the parent and that of selected offspring plants.

Selected deviating twin-halves are being evaluated and it is anticipated that crosses between contrasting low chromosome number genotypes could be made by 1993.

Promoter Analysis of the Pollen-Specific Gene NTP303 from Tobacco

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The isolation of a pollen-specific cDNA clone (NTPc303) from tobacco was reported previously (Weterings *et al.* (1992): *Plant Mol. Biol.* (in press)). The highly specific expression pattern of the gene NTP303 as determined by northern blot analysis has been corroborated by in-situ localization using confocal laser scanning microscopy. Transcription of the NTP303 gene starts late during development of the pollen grain and continues during pollen tube growth. No homologous transcripts can be detected in vegetative or other flower tissue. Combined with the fact that NTP303 is expressed in a broad variety of pollen species, these results suggest that NTP303 codes for an indispensable function in pollen. Nucleotide sequence analysis and database searches however, have not come up with a possible function for this gene. Homologous pollen-specific cDNA clones have been isolated, however: LAT51 from tomato (S. McComick) and Bp10 from *Brassica* pollen (S. Fabijanski) (pers. comm.).

The high and very specific expression characteristics of NTP303 make this gene a suitable object for studying the regulation mechanisms involved in tissue-specific gene expression. A genomic clone (NTPg303), homologous to NTPc303 was therefore isolated. The restriction map of the clone NTPg303, when compared to the NTPc303 map suggests an intronless gene. This suggestion is confirmed by the preliminary sequence results.

Using the 5′ coding region of the NTPg303 clone, it has been determined that three copies of NTP303 are present in the genome of tobacco. Also homologous genes have been found in the genomes of *Petunia*, tomato and *Arabidopsis*.

In order to be able to study the promoter of a gene, first the transcription site of that gene has to be determined. The region of the start of transcription of NTP303 has been roughly determined by northern blot analysis using two different probes: F9-3 containing the 5′ region −700 to −77; F9-32 containing −700 to −199 (relative to the start ATG). As F9-3 could and F9-32 could not generate a hybridization signal, the transcription-start site was determined to lie between positions −77 and −199. An RNase protection assay using F9-3 as a probe further delineated the transcription-start site to position −152 (relative to the start ATG).

Interestingly the TATA box most proximate to the transcription-start site is highly homologous to the TATA region of LAT52 (a pollen-specific gene from tomato) and Bp10 (a pollen-specific gene from *Brassica*). Nucleotide sequence analysis of the first 500 bp upstream of the coding region of the gene has resulted in the identification of three core motifs of the *cis*-acting element conferring pollen specificity (GTGG or GTGA). An extended region surrounding one of these motifs was 100% homologous to the pollenbox (TGTGGTT) and 52/56-box (TGTGGTTAATATA) as postulated by D. Twell. The functionality of these regions will be determined in future research, targetting these regions for mutagenesis and subsequent transformation of pollen and/or plants.

MEETING OF THE NETHERLANDS SOCIETY FOR PLANT CELL AND TISSUE CULTURE SPRING 1992

Anther and Microspore Culture of *Hordeum vulgare* cv. Igri

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The influence of environmental and culture changes was investigated on both anther culture and microspore culture of barley cv. Igri. The highest regeneration frequency for both culture systems was obtained when at least 50% of the microspore population was in the mid-late to late uninucleate stage, when the anthers were pretreated for 4 days on mannitol and when culture was performed with oxygen supply at regular intervals. Furthermore, the supplement of vitamins and casein hydrolysate in the culture medium improved the microspore culture, whereas such supplement showed no or negative effect on the culture of anthers.

Anther culture is not as laborious as microspore culture, but turned out to be at least five-fold less efficient. When mechanically-isolated microspores were cultured, under the conditions found to be optimal in the present study, a mean of 12·4 green plants per anther was obtained. Consequently, microspore culture does meet the requirement of high regeneration frequency thus enabling application. Experiments aimed at stable transformation through particle bombardment of microspores, are in progress.

'Re-Invigoration' of Rose Rootstocks Following Micropropagation

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In the development of woody plants from seed there is a juvenile phase which, depending on the species, varies from several weeks to 30–40 years. When juvenile, flowering cannot occur under normal conditions. Once the ability to flower is achieved, the plant is considered adult or sexually mature. Before transition to maturity, vigour gradually decreases in most species. The reverse process, in which the plant regains juvenility is termed 'rejuvenation'; when only juvenile vigour is restored, this is called 're-invigoration'. Restoration of vigour brings with it improved adventitious root formation in some recalcitrant woody crops.

In the Dutch rose industry on artificial substrates, scion varieties are propagated onto clonal rootstocks by cutting–grafting, a method in which both the scion and the rootstock are shoot internodes. Normally stock internodes form adventitious root in 2–3 weeks time. Newly introduced clonal *R. canina* 'Inermis', that would be otherwise promising stocks, are notoriously recalcitrant. The possible re-invigoration following 24 months of micropropagation of six clonal rose rootstocks, including two 'Inermis' selections, was studied with regard to the rooting of softwood cuttings, which is comparable to cutting–grafting.

Under optimal conditions, cuttings of micropropagated source plants had heavier roots, more roots per cutting, larger total root length, larger

specific root length, and longer axillary sprouts than cuttings from source plants that were continuously grown in the greenhouse. In general, re-invigoration was more conspicuous as stocks were more recalcitrant under normal conditions, but also the well-rooting 'Multic' was evidently reinvigorated. Re-invigoration following sustained micropropagation may be conducive to cutting–grafting in other recalcitrant rose stocks.

2,4-D as a Signal Molecule in Plant-Cell Culture

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2,4-Dichlorophenoxyacetic acid (2,4-D) is used as a mitogen in plant-cell and tissue cultures. Its function is that of a single molecule, not of a nutrient.

In our laboratory, the role of 2,4-D in plant-cell division was studied using suspension cultures of tobacco cells. In standard LS-medium, cell division eventually stopped because of a concurrent lack of 2,4-D and phosphate. 2,4-D limitation appeared to result in accumulation of cells in the G2-phase of the cell cycle. Addition of auxin (2,4-D or NAA) induced cells to continue the cell cycle, whereas addition of cytokinin (kinetin or BAP) had no such effect.

The total amount of free 2,4-D remained constant during the growth period of the cells, but the distribution of 2,4-D among cells and medium changed. Apparently, 2,4-D is not lost due to breakdown or conjugation. 2,4-D transport might be a regulatory factor in control of plant-cell division. The first indications for a reduced uptake of 2,4-D by tobacco cells grown under phosphate-limiting conditions were found. The site of action of 2,4-D has yet to be determined.

The Flexibility of Plant Cells in Tissue Culture—Respiration and Growth

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Plant cells possess two respiratory pathways: the cytochrome pathway which is used preferentially, and the alternative, cyanide-resistant pathway. Respiration via the latter provides little energy (ATP) compared to respiration via the cytochrome pathway. The presence of the alternative pathway possibly enables the cell to react to situations in which the cytochrome pathway functions less or not at all.

This subject was studied by growing batch culture suspensions of *Petunia hybrida* in the presence of substances that suppress the cytochrome pathway.

Chloramphenicol (CAP) is an inhibitor of the mitochondrial protein-synthesis and therefore has an effect on a.o. cytochrome oxidase and ATP-synthase. Although the activity of cytochrome oxidase decreased, the cytochrome pathway mediated respiration of CAP-treated cells did not reveal much difference to that of control cells during the batch cycle. The employment of the alternative pathway increased significantly compared to control cells. The cells showed very little or no growth.

Growth of cells cultivated in the presence of Antimycin A (AA), an inhibitor of the cytochrome pathway mediated respiration showed a rather long lag-phase of about 1 week. After that, dry weight increased somewhat slower than in control cells and ended up at about 2/3 of control cells.

The AA-treated cells showed an immediate decrease in activity of the cytochrome pathway and the engagement of the alternative pathway was high. The presence of AA does not prevent the cytochrome pathway to increase again after 10 days. However, it seems unlikely that the cytochromal respiration is responsible for all ATP-formation and growth in the AA-treated cells; probably the alternative respiration takes part too.

It remains uncertain whether plant cells can grow on alternative respiration alone. Nevertheless the results indicate that the alternative pathway contributes to the physiological flexibility of the cells.

A Dramatic Influence of Embedding in Calcium Alginate on Protoplast Culture in Beet (*Beta vulgaris* L.).

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Plating efficiencies in protoplast cultures of *Beta vulgaris* L. (sugar beet, fodder beet) are generally low. In relation to our cybridization programme for *Beta* spp. we investigated a number of modifications to the basic protoplast culture protocol to identify means to improve in-vitro responses. Of these, the embedding of mesophyll protoplasts in thin, 1% calcium alginate discs resulted in the most dramatic improvement in cell development, in comparison to culture in standard liquid medium. Many more cells remained viable longer, resynthesized a cell wall and retained visible cytoplasmic activity. The greatest influence was, however, on cell division. In alginate cultures this occurred much earlier (after 2 days in contrast to

7 days in liquid medium), and at a significantly higher frequency (10–200-fold, dependent on genotype and tissue source) and lead to the formation of colonies which required transfer to fresh medium in half the usual time (18 days instead of 35 days). The regeneration capacity of the friable calli obtained from alginate cultures also appeared to be significantly enhanced although this requires confirmation. Attempts to identify the critical feature(s) of the alginate protocol which bring about these effects have lead to the conclusion that the alginate must be solidified and the cells must be embedded therein in order to realize the stimulatory influence of the technique. Agarose is not a suitable substitute for alginate in these experiments.

These results, and those reported for other systems, lead to the strong recommendation to test alginate embedding of protoplasts for all recalcitrant species in order to improve the cell-division response.

Hormone Sensitivity During the Successive Phases of Rooting in *Malus* Microcuttings

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Regeneration of roots occurs in three phases, namely dedifferentation, differentiation and outgrowth. We examined the timing of these phases in microcuttings of *Malus* 'Jork' rooted at 20°C with 1 μM indole-3-butyric acid (IBA). We assumed that the differentiation phase is inhibited by cytokinin. Pulses with 2 μM 6-benzylaminopurine (BAP) from 24 to 48 h or from 48 to 72 h strongly inhibited rooting, whereas 24-h pulses at other times had a much reduced effect. We therefore conclude that the differentiation phase lies between 24 and 72 h after excision of the microcuttings. Pulses with IBA given during the differentiation phase to microcuttings cultured on medium without hormones stimulated root formation. The promotive effect of IBA was not as distinct as the inhibitory effect of BAP and was only observed in pulses with a high concentration of auxin (3 μM). We conclude that the shoots have low-auxin requirement during the dedifferentiation phase to sustain competence, and high-auxin requirement during the differentiation phase to promote rooting. In discs excised from microcuttings and cultured at 25°C, the differentiation phase was somewhat advanced. In discs rooted under the same conditions, microscopical analysis has previously shown that the first cell divisions occur after 48 h. Thus, the differentiation phase coincides with the first cell divisions. We also examined the effect of 2,4-dichlorophenoxyacetic acid (2,4-D). This synthetic auxin strongly inhibited the outgrowth of roots, but not the dedifferentiation and differentiation phases.

Imaging of Cytosolic Ca^{2+} in Embryogenic Plant Cells by Confocal Microscopy

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Confocal laser scanning microscopy (CLSM) is a powerful tool for studying the distribution of free cytosolic Ca^{2+} in multicellular plant systems. In a CLSM equipped with an argon or argon-krypton laser, fluo-3 is a suitable Ca^{2+} probe. We used fluo-3 to examine the distribution of free cytosolic Ca^{2+} during plant embryogenesis.

Somatic embryogenesis of carrot is a well-known model system. In this system, embryos can easily be obtained in vast numbers by changing the culture medium from auxin-containing to auxin-free medium. Embryos arise from single cells present in the periphery of pro-embryogenic masses (pems) and proceed through the succeeding stages of embrogenesis: globular, heart shaped and torpedo shaped.

We were unable to load fluo-3 into carrot cells in its AM form or at low pH. Therefore we developed a protocol which included the use of 0·1% digitonin to permeabilize the plasma membrane (Fiskum, G. (1985): *Cell Calcium* **6:** 25–37). It appeared that carrot somatic embryogenesis coincides with a rise in the level of free cytosolic Ca^{2+}. In embryos from the heart to the torpedo shaped stage, a conspicuous signal was present in the protoderm. Intracellularly, the highest signal was observed in nuclei.

As embryogeneic cells normally develop into complete somatic embryos after dye loading (Timmers, A.C.J., Reiss, H.-D., Schell, J.H.N. (1991): *Cell Calcium* **12:** 515–521), we conclude that this procedure can be used to study the complete developmental process of somatic embryo formation by intravital microscopy.

A Model-System for Studying Cold-Induced Sweetening in Potato

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Cold-storage-induced sweetening is of considerable economic importance for the potato processing industry. During frying, the Maillard reaction between reducing sugars and amino acids causes unacceptable levels of browning. The susceptibility of potato tubers to low temperature is dependent on tuber maturity, environmental factors during tuber growth and, above all, varietal characteristics.

Cold-induced sweetening has been correlated with cold-lability of key glycolytic enzymes: ATP-phosphofructokinase (PFK) and/or PPi-phosphofructokinase

(PFP). To establish the contributions of these enzymes, transgenic plants were produced in which the corresponding genes had been altered. The investigation of the first group of transformants, derived from a readily transformable, diploid genotype (1024–2) expressing antisense *PFPβ*, is now in progress, as well as transformations of cold-sensitive target cv. Saturna.

Until now, the success of selecting improved genotypes with a reduced cold-susceptibility depended on cold-storage experiments of field-grown tubers. However, this procedure is time- and space-consuming and may be affected by seasonal and environmental factors. Therefore, the use of microtubers produced by in-vitro grown plantlets offers an attractive alternative. However, striking differences were observed between different genotypes for supplements required for optimal microtuber production. Supplements (and their concentrations) applied to the standard MS-medium were: sucrose (2–8%), jasmonic acid (JA, 0·01–100 μM), BAP (benzylaminopurine) or kinetin (1·0–2·5 mg l^{-1}), agar (0·8%) or Gelrite (0·2%). For the diploid genotype: 6% sucrose, 1 μM JA, plus 1 mg l^{-1} BAP were optimal and for cv. Saturna: 6% sucrose, plus ≥ 1 mg l^{-1} BAP. Surprisingly, the tuber-inducing substance jasmonic acid did stimulate microtuber formation in the diploid genotype, but not in cv. Saturna. Kinetin did not stimulate microtuber formation in 1024-2, whereas in cv. Saturna a positive effect was only observed in Gelrite-solidified media. Under the optimized conditions, more than 90% of explants formed microtubers in *c.* 4 weeks. In addition, it was demonstrated that cv. Kennebec microtubers showed sweetening during storage at low temperature. Glucose and fructose concentrations increased almost 10-fold to 1·0 g 100 g^{-1} f wt. in about 1 month of storage at 2°C (Claassen *et al.* (1992): *Potato Res.* (in press)). Similar results have been reported for field-grown tubers of this cultivar.

Initiation and Establishment of Morphogenic Cell Suspensions of Barley (*Hordeum vulgare*)

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The main aim of our research was to develop an efficient system for transformation and plant regeneration from barley cell suspensions.

Two methods for cell-suspension initiation were compared for seven barley genotypes. Callus was induced on immature embryos and, upon subculturing, fast-growing friable callus was selected. This selected callus was used to initiate cell suspensions. For the second initiation method, immature embryos were directly cultured in liquid medium.

An established cell suspension should (a) consist of small cell aggregates, (b) have a good growth rate and (c) have morphogenic capacity. A very important prerequisite for the regeneration of normal fertile plants from cultivated cells is the diploid status of the cells. Therefore, the ploidy level of nuclei isolated from the suspension cells was determined by flow cytometry.

With both initiation methods we were able to generate fast-growing cell suspensions consisting of small aggregates. If a suspension was initiated directly using immature embryos it took 2–3 months to establish a homogeneous cell suspension; with callus it took at least 5 months. At this moment we have established cell suspensions of four different genotypes.

The ploidy level of the cell cultures seemed to be dependent on the initiation method, tissue-culture time and the genotype.

For one genotype we were able to establish cell cultures with a high regeneration capacity. Approximately 50 green plants g^{-1} suspension cells were obtained. All regenerants were diploid and morphological normal. The first flowering plants were fertile.