Monitoring of *in vitro* cultured microspores switched to embryogenesis in *Olea europaea* L.

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Genetic improvement through conventional methods of breeding in woody plants involves many generations, and may take several years before any meaningful results are obtained and has still not given fruitful results. This has led to more emphasis on the use of *in vitro* techniques especially anther and microspore culture for genetic improvement of woody species like olive (*Olea europaea* L.). Olive tree is a woody and cultivated species, main product in Spain. The olive oil has a social importance and a good future due to consumer's necessities.

Searching for convenient morphological criteria to correlate anatomy of the flower bud with microspore developmental stage: For high frequency of haploid production, the appropriate stage of microspores is critical and it varies with species, and thus it is necessary to identify the appropriate stage of microspores for haploid plant production. (Bueno *et al.* 2002). To study the stage of the microspore in olive, directly from the trees growing in the field , its has taken branches with floral bud from the World-wide Collection Cultivars from Olive tree of Cordoba (Spain) in different zone of the tree. Numerous preparations with Aceto-Carmine and DAPI have been made, to be able to find parallelism between the morphology of floral buds and the stage of the microspore. Great asincrony has been observed, like in most of the woody species. Inside anthers extracted from flower buds with a similar size and color, microspores at uninucleate stage were obtained, whereas other floral bud with these same characteristics contained different stages, from tetrad until polen. During the gametophytic development, the initial stages of the material isolated from the anthers show very young microspores released from tetrads- after meiosis of the pollen mother cells- which are characterized by small size, weak autofloresecence of the wall under UV and a large and centrally located nucleus. In late-stage microspores, the nucleus is slightly condensed and located mostly near the polen wall. Microspore mitosis is asymmetric and produces nuclei that can be easily distinguished after staining with DAPI. Then, the generative nucleus is small, condensed and appears intensely stained; whereas, the vegetative nucleus is large, diffuse and appears weakly stained.

Monitoring in vitro formation of microspore-derived multinuclear structures: The sporophytic pathway *in vitro* will be determinated by the genotipe. When late vacuolate microspores or young bicellular pollen grains are cultured under stress conditions, they are switched from their naturally programmed pathway for gametophytic to sporophytic development. Then, these microspores start dividing in a different way than they do *in vivo*. There is a symmetric divisions with several nuclei 2,4,8...leading to embryoid or proembryo formation. The initiation of embryogenesis it is possible to observed in the multicellular structures that are in progress. The obtained pure lines, will be used for future genetic analysis and breeding programs. These results will have advantageous implications at scientific, social and economical levels.

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Use of gametic embryogenesis for *Citrus* improvement

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Citrus with 104,505,157 Mt in 2002 (FAOSTAT database) is the main world fruit crop. Breeding programs in *Citrus* aim to obtain new varieties with a shorter juvenile period, increased yield, a longer ripening season, regular bearing, improved fruit appearance and quality and, above all, without seeds.

Haploidy advantages and pollen biotechnology can represent a powerful tool in *Citrus* breeding (Germanà, 1997), with potential uses in mutation research, selection, genetic analysis and genetic transformation. Breeders and plant geneticists are also interested in obtaining homozygousity in woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, self-incompatibility. This interest justifies the need to conduct further research thereby increasing our knowledge of gametic embryogenesis with the goal of establishing efficient anther culture protocols in this important agricultural crop (Germanà, 2003).

Anther culture is one of the most interesting systems of plant regeneration through *in vitro* culture, and gametic embryogenesis is one of the most stricking examples of cellular totipotency (Reynolds, 1997).

Androgenesis has been successfully induced in genus *Citrus*, but only in few genotypes. Actually, the improvement of the induction rate and the increase of number of genotypes responding to androgenesis are important with respect to enhancing haploidy application in *Citrus* biotechnology and breeding. Although androgenesis research made great progress in recent years, several aspects of this phenomenon still remain unclear, particularly the induction process of androgenesis and the factors that control it. The identification of the inhibitory and stimulatory factors are fundamental especially for recalcitrant species like *Citrus*.

From anther culture of *Citrus* and its relatives, haploid, but also diploid and above all triploid calli, plantlets and plants have been obtained. Ploidy analyses of androgenic regenerants, enabled us to demonstrate that the largest percentage of them are triploids and not haploids or doubled-haploids as expected, opening the way to a fast new, innovative and promising tool to obtain seedlees triploids in *Citrus*. The importance of triploids in *Citrus* improvement stems from the seedlessness of their fruits; and the recovery of seedless varieties is one of the main goals in *Citrus* varieties breeding, because they are strongly required by the fresh fruit market.

Genetic analysis using isozyme analyses and microsatellite markers showed that the regenerants are homozygous and produced by gametic embryogenesis.

This report summarizes the current status of research on androgenesis in *Citrus*.

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Session a3

POLLEN FOR IN-VITRO PRODUCTION OF HAPLOIDS

The relationship between induction of embryogenesis and chromosome doubling in microspore cultures

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Chromosome doubling of haploid plants has been a major concern relative to their use in plant breeding, genetics, mutation and transformation. The ideal time to double the chromosome number would be the haploid uninucleate cell or microspore stage following meiosis. The microspore stage is most often used to induct embryogenesis for haploid plant production as it can provide a large number of partially synchronized cells. The uninucleate stage can be induced by stress or anti-microtubule agents to provide embryo induction. The slightly

older stage of during or just following the 1st mitotic division (PMI) of the microspore is also suitable for induction of embryogenesis. Embryo induction most often is accompanied by disruption of the polarized PMI. This disruption appears to be the key to both embryogenesis and chromosome doubling, depending upon the induction agent and both embryogenesis and chromosome doubling may be induced by the same treatment. The disruption of the 1st asymmetric mitotic division of the uninucleate microspore results in a symmetrical division producing two vegetative-type nuclei. These two nuclei often lie close together with no cell wall formed between them and that allows the nuclei to fuse through membrane coalescence (KASHA et al. 2001). Agents that disrupt the microfilament arrangement necessary for cell wall formation may also disrupt the spindle formation that separates the daughter nuclei. The method of doubling may be influenced by the concentration of the agents used. EADY et al. (1995) demonstrated that low dosages of colchicine induced a symmetric division of the microspores while higher concentrations blocked PMI. With complete spindle disruption, doubling may occur by endomitosis.

When an asymmetric PMI occurs during or prior to induction, the vegetative nucleus, which normally remains in the G1 cell cycle stage, begins to divide to form embryos. The generative nucleus may also undergo limited divisions but rarely continues to form embryos. Doubling can occur in such cells through nuclear fusion or endomitosis. KASHA et al. (2001) observed that chromosome doubling during mannitol treatment at room temperature appeared to be primarily by nuclear fusion. They also postulated that doubling by fusion probably occurs after a symmetric or an asymmetric PMI. Studies in both barley and wheat microspore culture treated with mannitol revealed that the wall did not form after nuclear division allowing the daughter nuclei to coalesce to produce homozygous doubled haploids. While not investigated, pretreatments during or after the PMI likely inhibit the wall formation so doubling could occur then or after a second division of the vegetative nucleus.

INDRIANTO et al. (2001) cytologically clarified the nuclear development of uninucleate microspores of wheat after a pretreatment with heat in media B, which contains mannitol that is also an inducing agent that is particularly effective in barley isolated microspore culture. LI and DEVAUX (2003) observed that a 7d mannitol pretreatment following a 21d cold pretreatment significantly improved chromosome doubling in barley microspore cultures. Many studies have now shown in a number of species that various microtubule inhibitors can induce good embryogenesis and also improve chromosome doubling. These show the typical symmetric first division of the uninucleate microspore, indicating that disruption of the microfibrils or microtubules in the cell can lead to both embryogenesis and chromosome doubling. Colchicine or its analogs tend to be the most effective for doubling but other microtubule antagonists such as trifluralin, oryzalin and amiprophos methyl (APM) are also effective in chromosome doubling when applied in the initial culture of the microspores. Such agents had previously been shown to be effective chromosome doublers when plants at various stages of development have been treated. Similarly, treatment of plants or microspores with 2-hydroxy nicotinic acid (2HNA) have led to good induction and doubling rates in wheat.

In summary, there appears to be a close correlation between some methods of induction of embryogenesis and the induction of chromosome doubling. Efforts to exploit this relationship are being investigated in many labs.

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LI, H., DEVAUX, P. 2003. High frequency regeneration of barley doubled haploid plants from isolated micarospore culture. **Plant Sci.** 164: 379-386.

Doubled haploids from oat (*Avena sativa* L.) anther culture

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Oat (*Avena sativa* L.) is one of the major cereals in temperate latitudes, since it favours humid cool climate and tolerates acidic soils. In Finland only barley is cultivated over larger area than oat. Oat yield is mostly

consumed as animal feed, but human consumption is tending to increase, because of nutritional benefits and healthy effects of oat grain. Worldwide, however, oat is a minor crop accounting for about 5-7% of world coarse grain production.

Haploids are defined as sporophytes with the gametophytic constitution. Chromosome number can double spontaneously or doubling can be induced by treating plantlets with antimitotic agents e.g. colchicine. Thus, completely homozygous doubled haploid (DH) lines can be obtained from a heterogenous crossing progeny in a single generation.

In cultivar breeding, use of DH-techniques saves time, since pure lines are derived quickly from the crossing progeny. Also the efficiency of selection for both qualitative and particularly quantitative traits is increased due to absence of dominance and heterozygosity of DH-lines. Possibility to obtain instant homozygosity provides valuable material also for genetic research purposes.

In oats, production of DHs has been reported through anther culture and through wide hybridization, but neither of these methods has yet been developed into a level where adequate rates of DHs could be produced for routine use (KIVIHARJU et al. 2000; Rines 2003). In this study we aimed at to increase the efficiency of oat DH-production *via* anther culture method. Several culture medium factors were tested on the basis of the previously reported protocol (KIVIHARJU et al. 2000). In addition, effect of light and temperature during induction phase were investigated. Significantly higher plant regeneration rates were obtained by optimized culture medium and conditions. In total, hundreds of oat DH plants were regenerated in these experiments.

Anther culture response is strongly influenced by the genotype. Introgression of desirable alleles by crossings from well-responsive genotypes to the recalcitrant ones can be used to enhance plant regeneration rates, as has been done in wheat, barley and rice. We used this approach successfully also for oat.

DNA markers associated with good anther culture characteristics, such as high ELS (embryo-like structure) production, high plant regeneration rates and large proportion of green plants, could be used to help the selection of crossing lines carrying favourable alleles for anther culture. Putative RAPD markers associated with improved anther culture traits were identified from the cross progeny of the recalcitrant oat and responsive wild red oat (*A. sterilis* L.) genotypes (KIVIHARJU et al. 2004).

As a conclusion, with the rates of DH-production reached in these studies, the efficiency needed for purposes of cultivar breeding and genetic studies is approached in some genotypes.

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Process of haploid formation in maize, barley, wheat and flax

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The value of haploids in genetic analysis and plant breeding has been known for a long time. However, haploids occur only rarely in nature. Induced androgenesis and gynogenesis are good source for haploid production in large number to be useful for breeding programmes.

We report on induction of haploids from anther and microspore cultures in maize, barley, wheat and flax.

In maize we described three pathways for androgenesis and have found the responsive stage for redirection of the developmental pathway /late uninucleate microspore/. The „star like" organization of maize microspores can be considered as a morphological marker of induced microspores. Induced maize microspore of used genotypes are bigger than 75µm and the spinul density on the microspore surface is less than in the rest of microspore population. The haploid response is a heritable trait so we tried to incorporate this trait into commercial maize lines by crossing with exotic ones.

In barley anther cultures the first microspore division was observed on the third day in culture and after ten days embryo-like structure appeared. The most responsive genotype was Vladan /15.38 structures per 100 anthers/ and we found 1.88 albinous per 100 anthers. We followed also the conditions influencing the albinotic plant occurrence.

In wheat we obtained better response if the donor plants were cultivated under field conditions in comparison with plants from the greenhouse. The androgenic response in 4 genotypes and 10 crossing combinations was compared and conditions for better yield of green plants were tested. Derived DH lines had very good performance in field conditions /good yield and good resistance against fungi diseases/ comparable to classical wheat lines.

In flax the effect of culture conditions and genotype were tested. The induction of callus formation from cultured anthers was the highest on N6 media with cultivar PR FGL 77 /12%/ and on N&N media with cultivar Carolin /2,8%/. Shoots were regenerated on N&N and N6 media supplemented with zeatin / 1mg.l^{-1}/ or BAP + NAA /1mg.l^{-1} each/. The highest number of shoots was regenerated with cultivar Red Wing.

Microspore-derived doubled haploids of wheat and maize: from fundamentals to breeding

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Nowadays cost saving agricultural production requires intensive wheat varieties with good nutrient responses, excellent quality and improved resistance to various environmental stresses. To achieve this goal modern wheat breeding utilises the results of plant biotechnology.

The present paper discusses key questions of the elaboration and perfection of anther/microspore culture techniques: cytological aspects of *in vitro* androgenesis, *in vitro* microspore selection and practical approaches.

The potential of doubled haploids in maize breeding has long been recognised.

Anther culture could be a useful tool for producing inbred lines possessing favourable agronomic characters. These can be used directly in heterosis breeding to construct new hybrids which satisfy market demands. However, the successful application of doubled haploid techniques in breeding is largely dependent on the androgenic capacities of the genotypes and on the frequency of induced or spontaneous chromosome doubling in plants of microspore origine.

During the last few years an efficient anther culture technique has been worked out in our laboratory. Culturability have been transferred from responsive, exotic germplasm (mainly of Chinese origin) into elit lines by crossing. Single cross hybrids in which the exotic parent represents 50%, or hybrids backcrossed to the elite lines (BC_1, BC_2) have been used as anther donor plants to develop new DH lines via anther culture.

This method makes it possible to produce a large number of microspore-derived embryos or calli capable of regenerating viable DH plants. Developmental processes taking place in anther cultures has also been studied in cellular and subcellular levels.

The efficient anther culture technologies elaborated for maize and wheat in our laboratory, have been applied to improve biotic and abiotic stress tolerance of these crops. *In vitro* microspore selection has been performed in wheat anther culture to produce aluminium tolerant doubled haploid genotypes (Barnabás et al. 2000). The improved aluminium tolerance of the selected DH1 and DH2 offsprings was also confirmed (Darko et al. 2002, 2004).

In maize anther culture *in vitro* selection was carried out for microspores of high androgenic capacity by using various oxidative stress stimulators (paraquat, t-BHP, menadione, riboflavin+ methionine). Haploid induction and plant regeneration capacity of the cultured microspores were affected drastically by the selective compounds. Ultrastructural features of the affected microspores were also studied. Irregularities in the nuclear structure and in the deposition of storage material in the cytoplasm could be detected. As the result of *in vitro* microspore selection: 15 paraquat-, 10 t-BHP-, 10 riboflavin- and 2 menadione tolerant fertile dihaploid plants could be developed. Further investigations will be carried out to test abiotic and biotic stress tolerance of the selected DH1 lines.

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Highly efficient double haploid regeneration in rye anthers *in vitro* cultures

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Regeneration of plants from microspores is an important stage on the way to genetic manipulations of plants. Regeneration of rye plants was described for different tissues and organs . Rye plants were regenerated through somatic embryogenesis from such explants as young inflorescence), leaf base and microspores. After many years of experiments regeneration of doubled haploids of rye is still a challenge and it is strongly genotype dependant process. In this study we were aiming at elaboration of the highly effective method of inducing androgenic callus and haploid embryos in order to apply this method for plant cloning, and genetic stabilizing as well as using somaclonal variation in plant breeding.

In vitro microspores can be induced to switch their development pathway from gametophytic - pollen grain formation, to sporophytic resulting in production of embryos. Plant material used in experiments should be morphogenic and make the regeneration of plants from microspores possible. For this very reason we have taken uninuclear microspores with considerable potency for cell divisions. Androgenic embryos formed from defined microspores or from callus transferred onto regeneration medium produced the plantlets after few days. The small plants were put into pots containing soil. After three months they matured in the greenhouse and set seeds. With these methods we were able to regenerate over 3000 of plants and over 2000 of them were fertile. Homozygous progeny of these plants will be used in rye heterosis breeding to establish new high yielding varieties.

An efficient system for regeneration of green plants from isolated microspores of hexaploid triticale

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Since its creation as a botanical curiosity, triticale (X *Triticosecale Wittmack*) has been transformed into a successful man-made crop with sizeable acreage. The potential of triticale, particularly as a forage crop, is promising. The diversity within its gene pool is an important asset for the future progress of classical and hybrid breeding. However, as with most crops, classical breeding approaches are relatively slow.

Recent advances in techniques of plant cell culture provide plant breeders with methods for production of homozygous lines from immature pollen grains - microspores. Isolated microspores can be induced to switch their development pathway from gametophytic to sporophytic, resulting in the formation of embryos and haploid plants. Spontaneous or induced doubling of chromosome numbers in these plants results in complete homozygosity and pure-breeding lines - doubled haploids (DH). Using this system, DH lines can be regenerated in a relatively short time compared to the conventional plant breeding methods, substantially reducing the breeding

cycle of a new cultivar. DH lines also have a potential in selection of recombinants, stabilization of transformed lines and in molecular mapping. The major problem with using DH is low efficiency of green plant regeneration.

The aim of this study was to develop a simple, efficient system of green plant regeneration of hexaploid triticale (*x Triticosecale* Wittmack) via direct androgenesis in the cultures of isolated microspores. The effects of various parameters such as growth regulators, microspore density and culture conditions were studied, leading to the development of an effective isolated microspore culture system. Induction responses for the combinations with growth regulators were not significantly different from controls for the initial divisions and for the numbers of the aggregates formed. The structures arising from microspores cultured with growth regulators showed less organized embryo formation and appeared inclined to form callus. The androgenic embryo yields on the hormone-free medium were up to ten times higher than in all remaining combinations combined. On high-density cultures the formation of the embryo-like structures was delayed, and that they did not develop directly into embryos or callus. In cultures where the microspores were cultured at relatively low densities, embryo-like structures sporadically produced embryos, but this reduced the overall regeneration efficiency. Cultures with the densities of 1.5×10^5 and 2×10^5 microspores/ml greatly had a markedly higher numbers of the microspore-derived embryos.

About 30% of the viable microspores were able to initiate the transition towards the sporophytic pathway within a few days of the culture initiation. Embryos possessed all structures typical for a normal zygotic embryos were formed from defined, individual microspores by direct embryogenesis. The first plants were regenerated about 21 days after the initial microspore isolation. The approach resulted in the regeneration of up to 54.9 green plants per single spike. The frequency of albino plants was about 15.8%. Among green progeny tested 30.8% were spontaneously doubled haploids; haploids represented 66.4% the analysed population. The remaining 2.8% of plants had ploidy levels different from haploid or diploid. High efficiency of green plant regeneration makes this system practical for accelerated cultivar development in triticale breeding programs.

Expression of zygotic embryogenic genes during androgenesis in *Hordeum vulgare* L.

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Barley haploid production has been achieved by both anther and isolated microspore culture (KASHA *et al.* 1990). A better understanding of the molecular and cellular events that take place in androgenesis will help not only to improve the yield and quality of haploids but also to obtain some clues about the early stages of the embryogenic process.

We have analysed in barley androgenesis induced using isolated microspore cultures according to Olsen (OLSEN 1991) the presence of the transcripts of three genes which are known to be expressed during zygotic embryogenesis. Using RT-PCR as well as *in situ* hybridisation reactions at light and electron microscope level we have compared their expression with that of gametogenesis and zygotic embryogenesis. Gene *END1* was selected because it has been reported as a molecular marker for the barley endosperm coenocyte (DOAN *et al.* 1996). The other two genes used were the homologous in barley to the Arabidopsis genes encoding N-acetyl-glucoaminidase (*Naglu*) and *EMB615*. These Arabidopsis genes were identified from a collection of mutants that present alterations in the early stages of embryogenesis.

We extracted total RNAs from different stages of gametogenesis, pollen and zygotic embryogenesis using the MRC TRI reagent kit according to the manufacturer´s instructions. With these RNAs we synthesised complementary DNAs using an oligo(dT) primer. Gene specific primers from the *END1* sequence and *Naglu*- and *615*-barley-homologous-EST sequences were then used to amplify the partial cDNA fragment by PCR. The PCR products of these genes were sequenced and cloned in pGEMT-Easy vector for *in vitro* transcription of the RNA probes with dig-UTP using T7 or Sp6 RNA polymerases. These dig-RNA probes were used for the *in situ* hybridisation studies at both light and electron microscope levels. For these experiments, samples from different stages of gametogenesis and microspore and zygotic embryogenesis were fixed in 0.25% glutaraldehyde and 4% paraformaldehyde in 0.5M cacodylate buffer, then dehydrated in ethanol series and embedded in Unicryl. Hybridisation was performed overnight at 45°C and visualised using a sheep antidig antibody coupled with 15nm gold particles for electron microscopy. For light microscopy we used the silver-enhancement technique.

RT-PCR results showed that the *END1* gene is not expressed during gametophytic development but it is expressed during microspore and zygotic embryogenesis. *END1* can be considered as a good marker for early embryonic development during androgenesis. The two genes homologous to those of Arabidopsis are expressed in both pollen and zygotic embryogenesis. The patterns of expression in gametophytic development and zygotic embryogenesis of *EMB615* and *Naglu* homologous genes are similar to those in Arabidopsis. Both genes are also expressed during early androgenic development. The results of the *in situ* hybridisation experiments are in accordance with those of RT-PCR and show the different spatial locations of the expression of these genes during gametophytic development, and pollen and zygotic embryogenesis.

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Androgenesis of *Lolium* x *Festuca* amphiploid cultivars in order to enhance gene expression for abiotic stress resistance

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Lolium multiflorum x *Festuca pratensis* amphiploid cultivars (2n=4x=28) are highly amenable to androgenesis. Large populations comprising primarily dihaploid genotypes with 7 *Lolium* and 7 *Festuca* chromosomes may be regenerated from a single parent genotype. The chromosomes of *Lolium* and *Festuca* pair and recombine at very high frequency which leads to considerable chromosomal rearrangements within each parental genotype and thereby a vast array of gametic variation. Each population of androgenesis-derived progeny represents the "dissected" genome of the parent genotype. The dihaploid progeny display an extreme range of both drought resistance and freezing tolerance, and genotypes with greater abiotic resistance than the parent are frequently recovered. Many dihaploid genotypes have sufficient male and/or female fertility to be used in a novel backcross breeding technology referred to as introgression-mapping. Despite the close homology of *Lolium* and *Festuca* genomes, their DNA can be distinguished cytologically using genomic in situ hybridisation (GISH). This enables us to locate the sites of intergeneric recombination. Alternative approaches may be used depending whether *Lolium* or *Festuca* (2x) species are used in the backcross breeding programme. Usually, stress-tolerant dihaploid plants are backcrossed onto *L. multiflorum* (2x). Introgressed *Festuca* genes for resistance against abiotic stresses are targeted by GISH. When after recurrent backcrossing, all but a single *Festuca* introgression have been removed from an otherwise complete *Lolium* genome, the introgression lines are assessed for their continued expression of abiotic stress resistance. *Festuca*-specific molecular markers are then applied to genotypes which retain stress resistance in excess to that found in the *Lolium* parent. Sequence tagged sites (STS) are then designed for use in breeding programmes and for cultivar development. The androgenesis-led technology has enabled better understanding of different mechanisms in *Lolium* and *Festuca* for acclimation of their photosynthetic apparatus to low temperatures. The transfer of a non-photochemical quenching (NPQ) mechanism for withstanding photoinhibition from *Festuca* to *Lolium* has led to enhanced freezing-tolerance. The advantages of targeting the signalling mechanisms for adaptations to abiotic stresses will be discussed.

Session a4

PALYNOMORPH WALL CHEMISTRY, STRUCTURE AND ASSEMBLY

Macromolecules in modern and fossil spores, pollen and algae

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Recognizable microfossils, such as pollen, spores and algae, in palynological slides have a long history of use, ranging from stratigraphic markers to entities that provide palaeoenvironmental and palaeoecological information. In most cases, the main underlying reason for their preservation in the fossil record is the fact that they are preserved as acid-resistant organic entities. However, this latter character may significantly affect the fossil record as differences in the chemical composition of these microfossils may bias against those that contain less resistant organic constituents. In order to understand and, possibly, correct for such biases, detailed insights into the chemical composition of both modern and fossil spores, pollen and algae is needed. This study presents an overview of the currently available literature information in combination with new molecular data from modern, sub-fossil and fossil microfossils, including spores, dinoflagellates, and other algae. The new data clearly reveal that the chemical composition of the fossil microfossils is in most cases not representative of that of the original biomacromolecules present in the modern counterparts and that significant chemical changes occur during fossilisation.

MALDI: a new technique with the potential to solve the sporopollenin enigma

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In the forty years of research into the chemical structure of sporopollenin, numerous chemical and instrumental techniques have been brought to bear on this complex biopolymer. Work using solid state ^{13}C- and ^{1}H- NMR and showed the presence of aromatic, aliphatic, and oxygen functionalities. Recently, pyrolysis and GC-Mass spectral analysis have elucidated a number of structural characteristics of sporopollenin. This GC-MS work suggests that two principle components of sporopollenin are fatty acids (C14-C16) and oxygenated cinnamic acid derivatives. We are currently investigating the use of Matrix-assisted laser desorption ionization (MALDI) mass spectrometry as a method of structure elucidation of sporopollenin. A relatively new technique, MALDI mass spectroscopy is a 'soft' technique used to ionize molecules up to several hundred kDa molecular mass. It is especially valuable in the detection and characterisation of biopolymers present in mixtures. We hoped to use this technique to gain insight on the component polymers of sporopollenin. Sporopollenin samples from *Selaginella* megaspores were analyzed following a number of treatment protocols, using different matrices. Additionally, we attempted to derivatise some of these samples by acetylation, followed by MALDI MS analysis. We report here our efforts to date.

Fluorescent staining procedure to identify pollen grains

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Palynological analysis is based on the identification of pollen grains through the distinguishing marks of the external structure, specifically openings and sculptures. Therefore, to point out the exine features, it is often necessary to use acetolysis or dyes.

Acetolytic method destroys all the pollen material with the exception of the exine, this emphasize external structures but may cause misleading in the functional interpretation of some pollen types (HESSE & WAHA, 1989). It is also reported that over-acetolysed grains appear often crumpled and distorted and structural information may be lost (DAFNI, 1992). As regards dyes, several procedures are available in order to stain the exine (KEARNS &INOUYE, 1993).

Observing with light microscope, the internal structure of grains, especially intine and cytoplasm, obstruct a free vision of the sculptures of exine. As a consequence, it results sometimes difficult, or even impossible, to unambiguously characterize small details Therefore, techniques based on light microscopy are sometimes insufficient to detect small details of the external structure of pollen. To overtake this limit several scientists have promoted the scanning electron microscope (SEM) and confocal laser scanning microscope as a necessary tool to observe fine details on pollen surface useful for diagnostic purposes (LANGFORD, 1990; RONNEBERGER, 2002).

Fluorescence microscopy is applied to anatomical and cytological studies of many biological structures because it is much more sensitive than its white light counterpart as a result of the enhanced contrast generated when a light object is viewed against a dark field. Moreover, since fluorescence microscopes are generally combined with a transmitted white light, the same field of a specimen can be observed subsequently with both methods. With this technique, information can be gathered from the two microscopes with an additive process and in a very short time.

The aim of this study was to combine traditional methods of pollen histochemical staining with fluorescence microscopy techniques in order to obtain clearer images of the exine. The methodology proposed herein provided images with enhanced contrast where the sole exine is evidenced without the interference of cytoplasm and pollenkitt, allowing the unambiguous detection of fine details of the exine.

Pollen was collected from anthers at dehiscence and several tests were performed in order to find the best staining and mounting medium for slides. Basic fuchsine stain in glycerol jelly (DAFNI, 1992) was selected. The slides were then observed through an epi-fluorescence microscope. The band pass filter, the dichromatic mirror and the barrier filter of the fluorescence microscope were appropriately selected to get clear view of fluorescent exines in dark background. With this method, the possible presence of fungi and debris on the slide does not interfere with pollen view because they appear not fluorescent.

Our results showed that the combination of staining procedures with fluorescence microscopy is a straightforward method that adds a clear view of fine details to the traditional observation of pollen morphology, without requesting complex preparation of the samples compared with acetolysis or SEM processes. Since this technique is neither costly nor time consuming, its use is very promising in the laboratories of melissopalinology, paleopalinology, aerobiology where routine analyses for pollen classification are performed.

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