Acta Bot. Neerl. 42(3), September 1993, p. 319–339

Pyrolysis mass spectrometry of developmental stages of maize somatic embryos

ANNE MIE C. EMONS*, MARCEL M. MULDER‡ and HENK KIEFT

Department of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands and ‡FOM Institute for Atomic and Molecular Physics, Unit for Mass Spectrometry of Macromolecular Systems, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

SUMMARY

The diversity in biopolymers such as polysaccharides, (poly)phenols, nucleic acids and lipids in the developmental stages of two types of histologically different somatic embryos of maize was investigated, using in-source pyrolysis low voltage electron impact mass spectrometry (PyMS) and the data were compared with those obtained for zygotic embryos. The first type of somatic embryo is attached to callus tissue and shows the same histo-differentiation characteristics as zygotic embryos. The second type grows from clusters of cells in liquid culture and its shoot meristem is blocked in development (Emons & Kieft 1991).

In-source pyrolysis low voltage electron impact mass spectrometry shows that during callus initiation from young zygotic embryos the relative abundances of di- and triglycerides decrease, but increase again after establishment of the embryogenic callus. During callus initiation mass peaks for ferulic acid appear. The embryogenic cell clusters contain more pentosans and hexosans than the non-embryogenic elongated cells. In the globular stage, the hexosans increase. At the ovoid stage, when the first cells with vascular thickenings are observed in the sections, which are brightly fluorescent and can be stained with phloroglucinol, mass peaks for lignin are observed in the mass spectrum. The mass spectra of the developmental stages of somatic embryos grown attached to callus are quite comparable to those of zygotic embryos. Both structures contain far less phenolics, no lignin-like polyphenols, and a much higher amount of polysaccharides than the single somatic embryos.

Key-words: histology, pyrolysis mass spectrometry, somatic embryogenesis, suspension culture, *Zea mays*.

INTRODUCTION

Regeneration *in vitro* is a prerequisite for obtaining progeny after genetic manipulation. As somatic embryos have one or only a few cells retraceable to the origin, the

*To whom correspondence should be addressed.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; PBS: Phosphate buffered saline; PyMS: Pyrolysis mass spectrometry.

regeneration of somatic embryos via single embryos is to be preferred above that of embryos grown in clumps attached to callus tissue. Regeneration of maize via somatic embryogenesis is only possible if the developing somatic embryo is, or has been, attached to a piece of callus tissue (Vasil & Vasil 1986; Rhodes *et al.* 1988; Emons & Kieft 1991). Also, the reported attempts of regeneration of protoplasts derived from suspension cells require a callus phase (Prioli & Sondahl 1989; Shillito *et al.* 1989). Though somatic-embryo development attached to callus can occur in liquid culture, the process is more efficient on solid culture media (Emons & Kieft 1993). Single somatic embryos, however, can only be obtained in liquid medium, but their development at the shoot side is blocked (Emons & Kieft 1991). Knowledge of the molecular differences of the two types of maize somatic embryos, single but non-regenerable and attached to callus but regenerable, might provide clues for regeneration protocols.

Using analytical pyrolysis mass spectrometry (PyMS), fingerprint information of various component classes of a small, solid sample can be obtained. This technique has been shown to provide a means of mapping (bio)molecules such as (poly)phenols and polysaccharides in biological material (Boon 1989; 1992). PyMS is based on flash heating of a sample, leading to a volatilization of smaller units. These compounds are ionized using low voltage electrons (16 eV). Ionized fragments are accelerated and mass separated in a mass spectrometer. The mass spectrum thus obtained provides information which can be related to the compounds present in the sample.

The aim of this research is to correlate histological and pyrolysis mass spectrometric data to developmental stages of two types of maize embryos during differentiation, one with the potential to form plants, the other blocked in shoot meristem formation, and to compare these data with similar data from zygotic embryos.

MATERIALS AND METHODS

Culture

Embryogenic callus, derived from zygotic embryos of maize genotype 4C1 (developed by Sandor Mórocz and Dénes Dudits, Cereal Research Inst., Biol. Res. Centre, Szeged, Hungary, Mórocz *et al.* 1986) was subcultured biweekly on 0·7% agar-solidified N6 medium (Chu *et al.* 1975), containing 2 mg litre^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D), 20 mM L-proline, 200 mg litre^{-1} casein hydrolysate, 2% sucrose and 3% mannitol, the proliferation medium, at 24–26°C under 16-h light/8-h dark (Emons & Kieft 1991). To obtain fully developed somatic embryos from callus aggregates, the aggregates were transferred for 10 days to agar-solidified medium (Murashige & Skoog 1962) with 6% sucrose, the maturation medium. During the maturation phase, samples were taken every day (T_0–T_{10}).

Suspension cultures were initiated from friable embryogenic callus aggregates 1 week after subculture at a concentration of 2-g fresh weight 50-ml^{-1} medium in proliferation medium without agar in 250 ml flasks in the same light conditions. The batch cultures were shaken at 100 rpm on a gyratory shaker and subcultured weekly.

Single somatic embryos from suspension culture were collected by sieving the suspension cells through sieves having pores of 50, 125, 212 and 300 μm, which resulted in samples containing primarily elongated cells, but also single cells and clusters of isodiametric cells (50- and 125-μm sieve), primarily cell clusters (212-μm sieve), and small globules (300-μm sieve). From a young culture a sample containing elongated cells

only was obtained. Ovoid and three polar (1·5-, 3- and 5-mm long) stages from cultures in which the maturation process had been started, were manually selected. These somatic embryos could not be further developed in or on maturation medium, and obtained a root only in or on regeneration medium.

Histology

Samples were fixed in 3% glutaraldehyde in PBS buffer, washed and dehydrated in a graded series of ethanol and embedded in Technovit 7100. Sections were stained with toluidine blue or with potassium iodide. Autofluorescence was determined (excitation wavelength 365 nm), and non-embedded sections were stained with 1% phloroglucinol in 15% HCl for detection of lignin.

Pyrolysis mass spectrometry

Before PyMS analysis all samples were washed five times in demineralized water to remove any medium constituents. Analytical in-source PyMS was performed on a JEOL DX 303 double focusing mass spectrometer (E/B mode). Data were collected on a JEOL DA 5000 system which was used without modification, transferred to a Sun Sparc IPC workstation (Mountain View, CA, USA) and processed using the Kratos analytical Mach3 software package (Kratos, Manchester, UK). A homogenized suspension of the sample (5-μg solid material) was placed on the platinum/rhodium wire (0·1-mm dia., 10% Rh) of an insertion probe, dried under vacuum and introduced directly to the ion source of the mass spectrometer. Desorption and pyrolysis fragments were ionized with 16 eV electrons and accelerated with 3 kV. The mass range was set to 20–1000 Da with a scan time of 0·6 s (total cycle time, 1·0 s). The temperature of the probe was increased at approximately 15°C s^{-1} to a final temperature of 800°C; the temperature of the ion source was 180°C.

Principal components analysis

Principal components analysis was performed on time-integrated PyMS data. Spectral data were transferred to a Sun Sparc IPX workstation (Mountain View, CA, USA) and processed with FOMpyroMAP, a modified version of the Arthur package (1978 edition, Infometrix, Inc., Seattle, WA, USA).

RESULTS

Different stages of maize embryo development were studied histologically and analysed with pyrolysis mass spectrometry. In Figure 1 a schematic representation is given of the callus formation and regeneration cycle starting from immature zygotic embryos. The numbers in the figure refer to specific developmental stages and these numbers will be used throughout this paper. Development from immature zygotic embryos (11) proceeds through callus (1), pro-embryogenic masses (3), globules (4) and ovoids (5) into the polar stage (6, 7) and stops at this latter stage. A root meristem is present and a root will develop but this embryo is blocked in its shoot meristem. If the intermediate stages (2–7) are cultured in the presence of 2,4-D, an embryogenic callus is formed again. The somatic embryos that grow attached to callus cells may further develop into new mature plants, just as the original callus (1) does.

Pyrolysis mass spectrometry provides fingerprinting information on the various compound classes in the sample. Some of these compound classes together with some

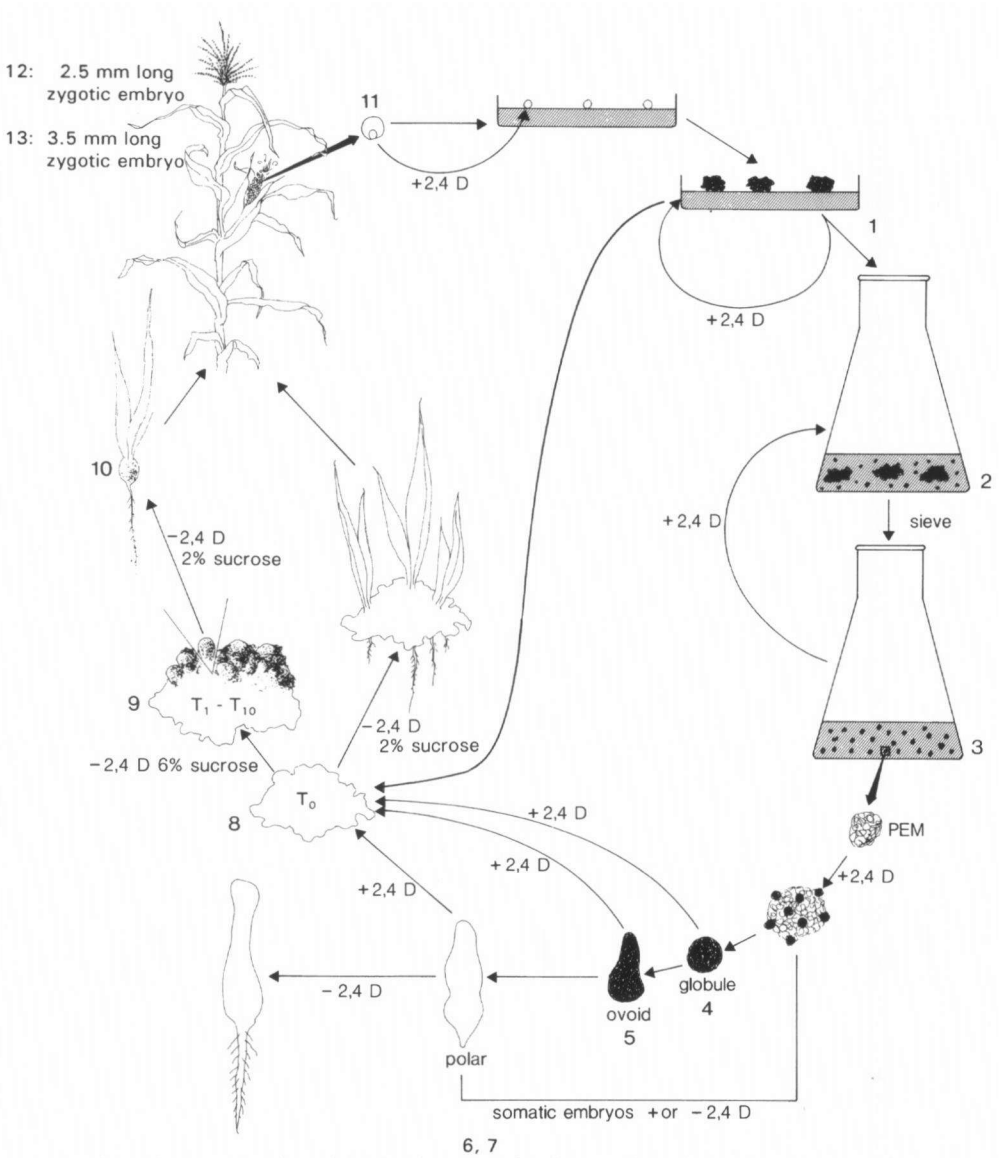

Fig. 1. Schematic representation of the development of maize zygotic embryos into somatic embryos which may or may not further develop into new plants; PEM: pro-embryogenic mass; T_0, T_1, etc: days of embryo maturation starting from embryogenic callus ($=T_0$).

characteristic mass numbers are presented in Table 1. These mass numbers represent molecular ions of compounds derived from the sample but can also be assigned to fragment ions of these compounds formed during low-voltage electron impact ionization. These fragment ions are intrinsic to the applied method of ionization. During sample heating different events take place: at relatively low temperatures (30–350°C) desorption takes place and at higher temperatures (350–800°C) pyrolysis of polymers occurs (Boon 1992). The mass spectra presented in this paper are summed and averaged over the temperature range 160–800°C.

Table 1. List of pyrolysis low-voltage electron-impact mass peaks for maize embryos

Compound	Mass peaks (*m/z*)
Polysaccharides	
hexose polysaccharides	126, 144
pentose polysaccharides	114, 85
Lignin	
guaiacyl fragments	180, 178, 166, 164, 152, 150, 137, 124
syringyl fragments	210, 208, 196, 194, 182, 180, 167, 154
Phenolic acids	
p-coumaric acid	120, 147, 164
ferulic acid	150, 177, 194
Triglycerides	830 $((C_{16:0})_2/C_{18:2})$; 854 $(C_{16:0}/(C_{18:2})_2)$; 856 $(C_{16:0}/C_{18:1}/C_{18:2})$; 878 $((C_{18:2})_3)$; 880 $(C_{18:1}/(C_{18:2})_2)$; 882 $((C_{18:1})_2/C_{18:2})$
diglyceryl unit	550 $((C_{16:0})_2)$; 574 $(C_{16:0}/C_{18:2})$; 598 $((C_{18:2})_2)$; 600 $(C_{18:1}/C_{18:2})$)
fatty acyl unit	256, 262, 280
Fatty acids	228, 256, 284, 282, 280
Sterols	414, 412, 396, 394
Nucleic acids	111, 112, 126, 135, 151
Proteins	91, 92, 94, 100, 108, 117, 131, 138, 152, 154, 166, 174, 176, 178, 186, 188

References to Table 1: polysaccharides, lignin, fatty acids, Mulder *et al.* (1991b); phenolic acids, Mulder *et al.* (1992); sterols, proteins, nucleic acids, Niemann *et al.* (1992); triglycerides, Wood (1980).

Callus initiation from zygotic embryos

Immature zygotic embryos of maize (11) that possess a shoot meristem and a developing scutellum produce embryogenic callus, if placed on agar-solidified N6 medium supplemented with 20 mM L-proline, 200 mg litre^{-1} casein hydrolysate and 2 mg litre^{-1} of the synthetic growth regulator 2,4-D. This callus type (1), called friable, consists of meristematic areas on the outside of the callus, comparable to globular stage embryos (Fig. 2a), attached to each other via vacuolated callus cells (Emons and Kieft 1991). On maturation medium the globular stage somatic embryos mature by developing a root and a shoot meristem and a scutellum (Fig. 2b), the storage organ from which the seedling draws food and which, in zygotic embryos, absorbs food stored in the endosperm. The mature somatic embryos also, if detached from the callus (Fig. 2c), develop into plantlets (Fig. 2d).

In Fig. 3a the mass spectrum of a 1·5-mm long zygotic embryo (11) is shown, revealing mass peaks for triglycerides $((C_{16:0})_2/C_{18:2})$; $(C_{16:0}/(C_{18:2})_2)$; $(C_{16:0}/C_{18:1}/C_{18:2})$; $(C_{18:2})_3$; $(C_{18:1}/(C_{18:2})_2)$; $((C_{18:1})_2/C_{18:2})$ and diglyceryl fragment ions $(C_{16:0})_2$; $(C_{16:0}/C_{18:2})$; $((C_{18:2})_2$; $(C_{18:1}/C_{18:2}))$ (lower panel). In the lower mass range mass peaks are observed for $C_{18:2}$ fatty acyl groups and C_{16} fatty acid (upper panel). The diglyceryl and fatty acyl groups are probably formed during ionization from the triglycerides. Mass peak *m/z* 313 is a fragment ion of a triglyceride consisting of a C_{16} fatty acyl group and a glycerol residue. The di- and triglycerides as well as the fatty acyl groups will be referred to as lipids in this paper. Also in the mass spectrum, characteristics for sterols such as stigmasterol and to a lesser extent β-sitosterol are found. The structure of

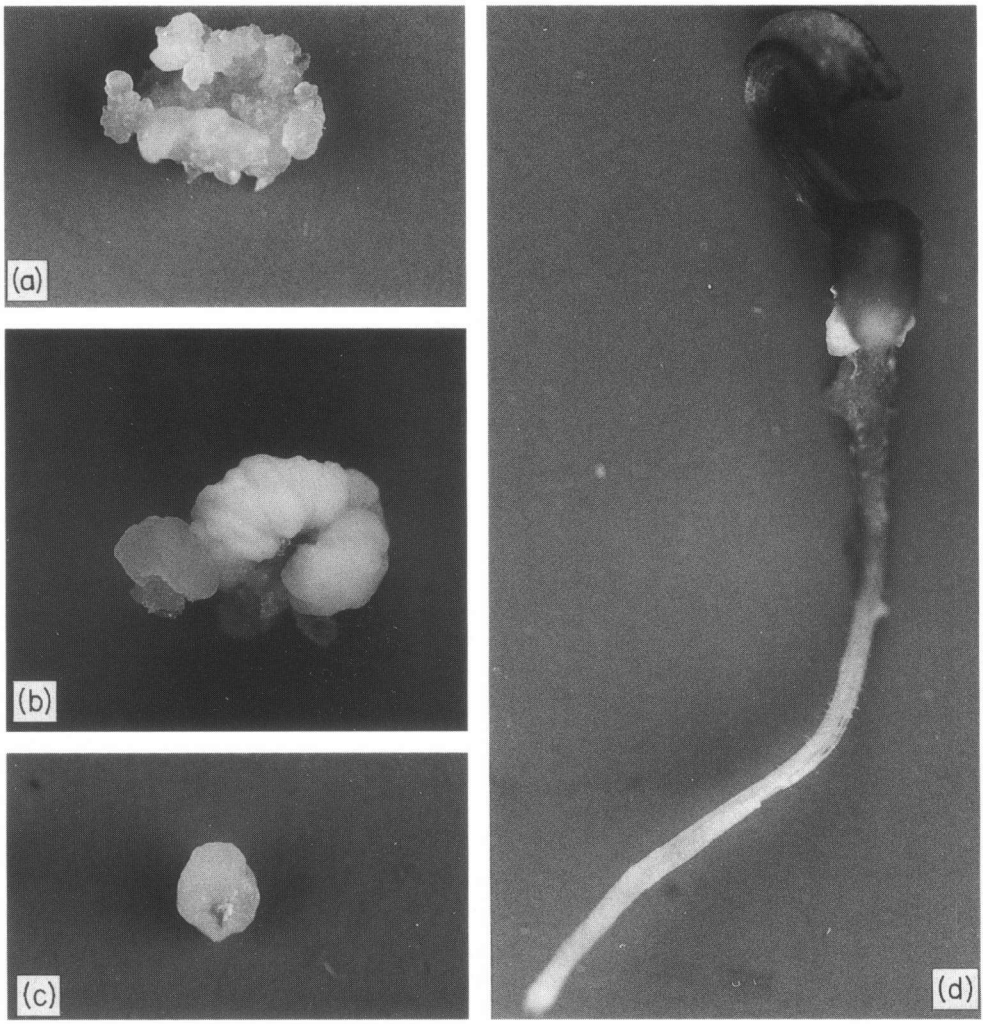

Fig. 2. Photographs of: (a) young embryogenic (=friable) callus, (b) embryogenic callus after 10 days on embryo-maturation medium, (c) single somatic embryo initially grown attached to callus aggregate showing scutellum and embryo-axis, (d) germinating plantlet from somatic embryo. Magnification: 10 ×.

stigmasterol is analogous to β-sitosterol with an additional double bond at the C_{22} position. At mass *m/z* 396, β-sitostene, a dehydration product of β-sitosterol is found. Similarly, a dehydration product of stigmasterol is observed at mass *m/z* 394. At lower masses, mass peaks for polysaccharides are observed. The relatively high abundance of mass peaks *m/z* 111 and 135 point to the presence of, respectively, cytosine and adenine. It is likely that thymine adds to the relative abundance of mass peak *m/z* 126. The sample also contains a high proportion of protein which is observed in the mass spectrum as a densely populated mass spectrum in the mass region between *m/z* 100 and 400.

In the mass spectrum of a 2·5-mm long zygotic embryo (12) (Fig. 3b) the relative abundance of hexosans is high compared to that of the 1·5-mm long zygotic

embryo (11). No mass peaks for (poly)phenols are observed. Lipids are abundantly present.

In Fig. 3c the mass spectrum is shown of a callus-producing zygotic embryo (1) which was 1·5 mm long at the beginning and then placed for 3 weeks on callus initiation medium. The relative abundance of the lipids has decreased during callus initiation; in the spectrum the reduction of fragment peaks for acyl-groups can be observed. In this sample, high abundances of ferulic acid and of sugars are observed. The relative abundance of hexosans is decreased compared to the abundance of pentosans.

After 2 months of proliferation on callus maintenance medium, the embryogenic callus (1) shows again a prominent mass peak for $C_{18:2}$ fatty acyl groups at *m/z* 262 (Fig. 3d). The relative abundance of lipids is increased. This is observed in the mass spectrum by an increase in $C_{16:0}$ glyceryl fragment peaks. In this established embryogenic callus only a small amount of ferulic acid is observed. The relative abundance of hexosans and pentosans is similar in this sample.

Callus transfer from solid to liquid medium

Histological analysis of a large number of calli shows that the globules constituting the calli grown on solid media, have a protodermal cell layer with the outer cells adhering tightly to each other, while those grown in liquid medium are covered with loosely adhering cells which are continuously sloughed off from the callus aggregates (data not shown). Both calli have no or few vascular elements. Figures 4a, b show mass spectra of embryogenic callus grown on solid medium for 2 months and 43 days (1) (a) and embryogenic callus grown for 2 months on solid medium and after that in liquid medium for 43 days (2) (b). In the spectrum from embryogenic callus in liquid medium a higher relative abundance of nucleic acids is observed. Both samples have a similar relative abundance for lipids and only small quantities of ferulic acid. The relative abundance of hexose and pentose polysaccharides for the cells in liquid culture is lower than for the cells on solid medium.

Sieve fractions of suspension cells

Two types of cells are sloughed off from the embryogenic calli in liquid culture: elongated cells which grow very fast and become very large but do not divide, and isodiametric cells and clusters of isodiametric cells which are able to divide (Emons & Kieft 1993). Young suspension cultures produce elongated cells solely (Fig. 5a). Different cell types can be separated from each other by size using sieves with different mesh sizes: a fraction between 45 and 125 μm consisting of single cells and small clusters (3) (Fig. 5b); a fraction between 125 and 212 μm containing clusters of isodiametric cells (3) (Fig. 5c); and a fraction between 212 and 300 μm consisting of large clusters and small globules (4) (Fig. 5d). Because elongated cells vary in size, all sieved samples are contaminated with those cells.

Elongated cells have large vacuoles and few, small starch grains, isodiametric cells (3) have smaller vacuoles, more starch grains and a higher cell wall/cell content ratio. The globules (4) show this tendency even more and protoderm may be formed.

The mass spectra of the various sieve fractions resemble each other. All fractions are characterized by minor quantities of lipids. In all sieve fractions mass peaks for ferulic acid are observed. The mass spectrum consisting of primarily small globules (sieve fraction 300 μm (4)) (Fig. 6b), is very similar to the 125-μm and 212-μm sieve fractions

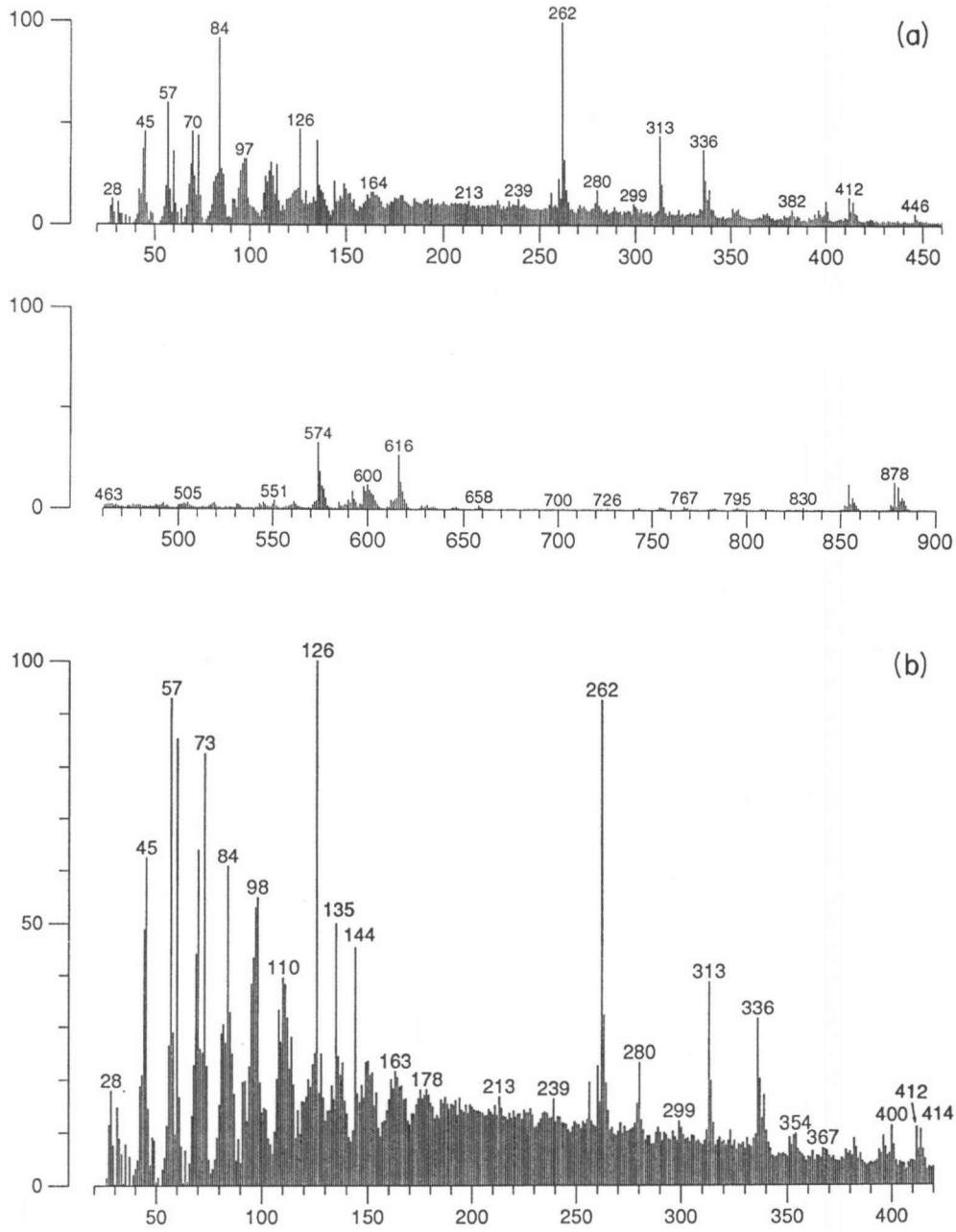

Fig. 3.

with respect to the polysaccharides; the less than 45-μm fraction resembles the elongated cells fraction (Fig. 6a) (data only shown for elongated cells and the 212–300-μm sieve fraction). The hexose/pentose ratio is higher in the 300-μm fraction than in the 45-μm fraction.

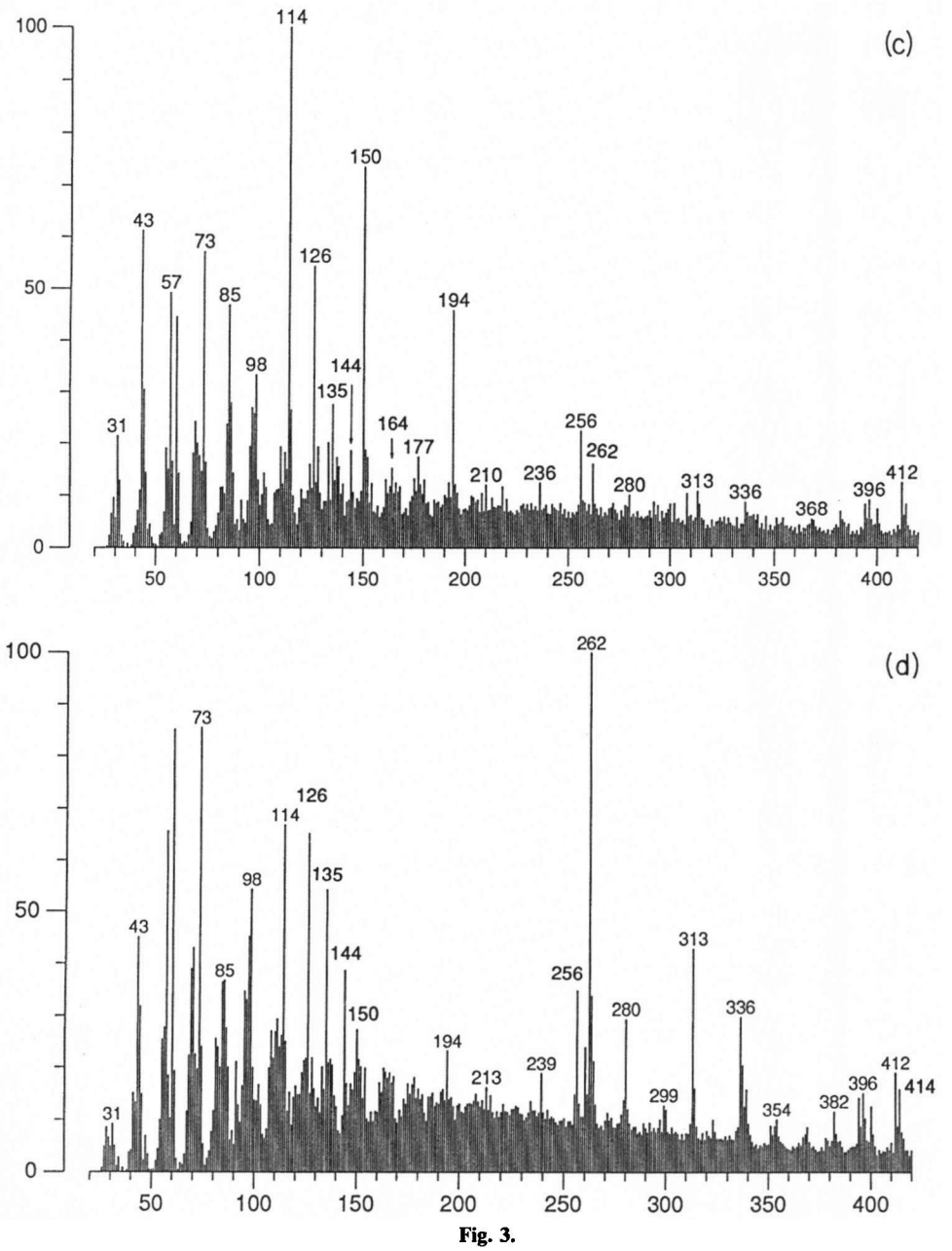

Fig. 3.

Fig. 3. Pyrolysis mass spectra of: (a) zygotic embryo 1·5 mm long, (b) zygotic embryo 2·5 mm long, (c) 1·5 mm long zygotic embryo producing friable callus, (d) proliferating friable callus.

Single somatic embryos

Apart from suspended cells and callus aggregates the liquid culture contains globules (4), ovoids (5) and polars (6). They represent developmental stages of young single somatic embryos. The ovoids (5) (Fig. 7a) and polars (6) contain a root meristem on one

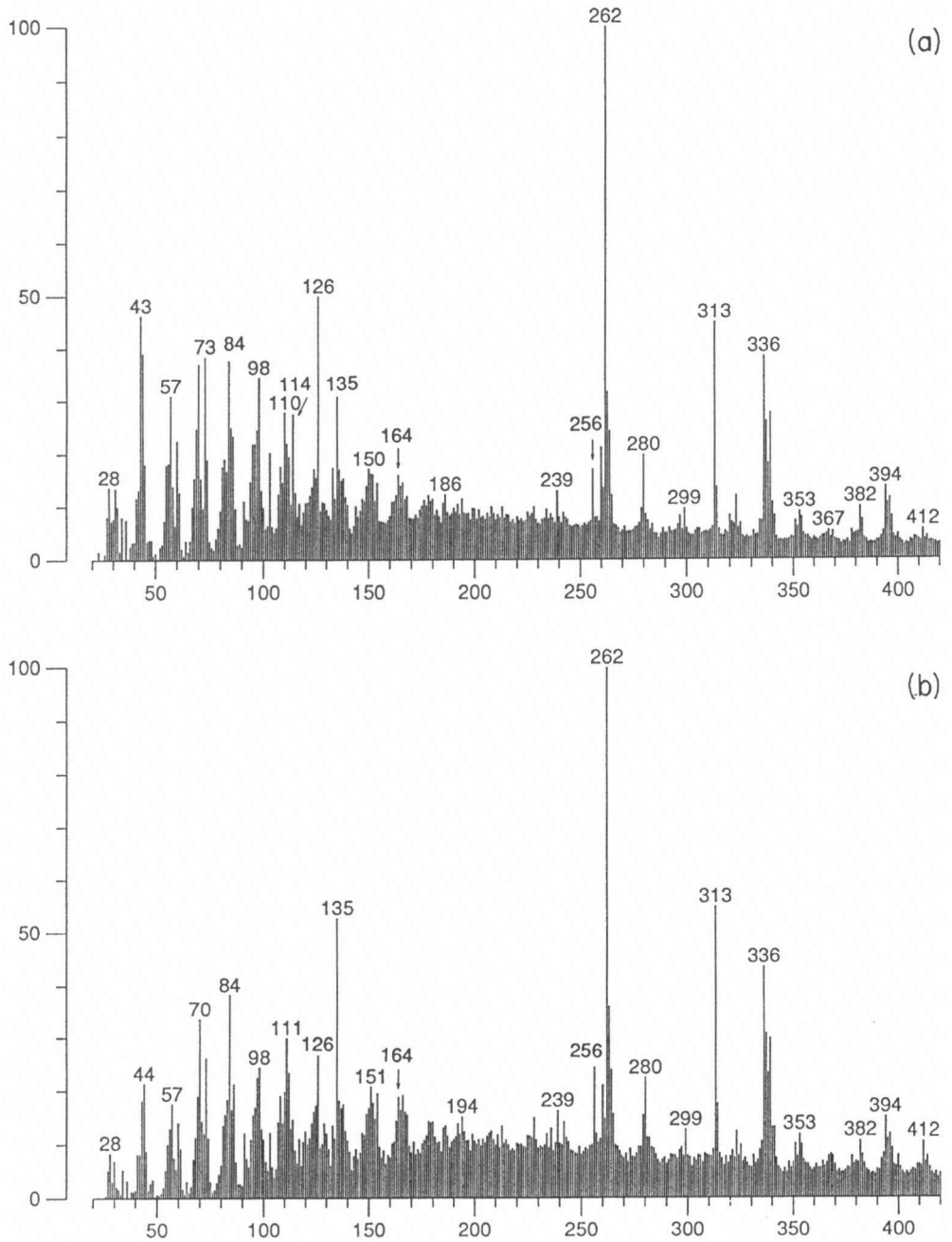

Fig. 4. Pyrolysis mass spectra of: (a) proliferating friable callus on agar plate, (b) proliferating friable callus in liquid medium.

side and the polar stage contains a meristematic zone on the opposite side with connecting cells in between but no scutellum (Emons and Kieft 1991), the storage organ of this type of monocotyledonous embryos. This spatial organization represents only the

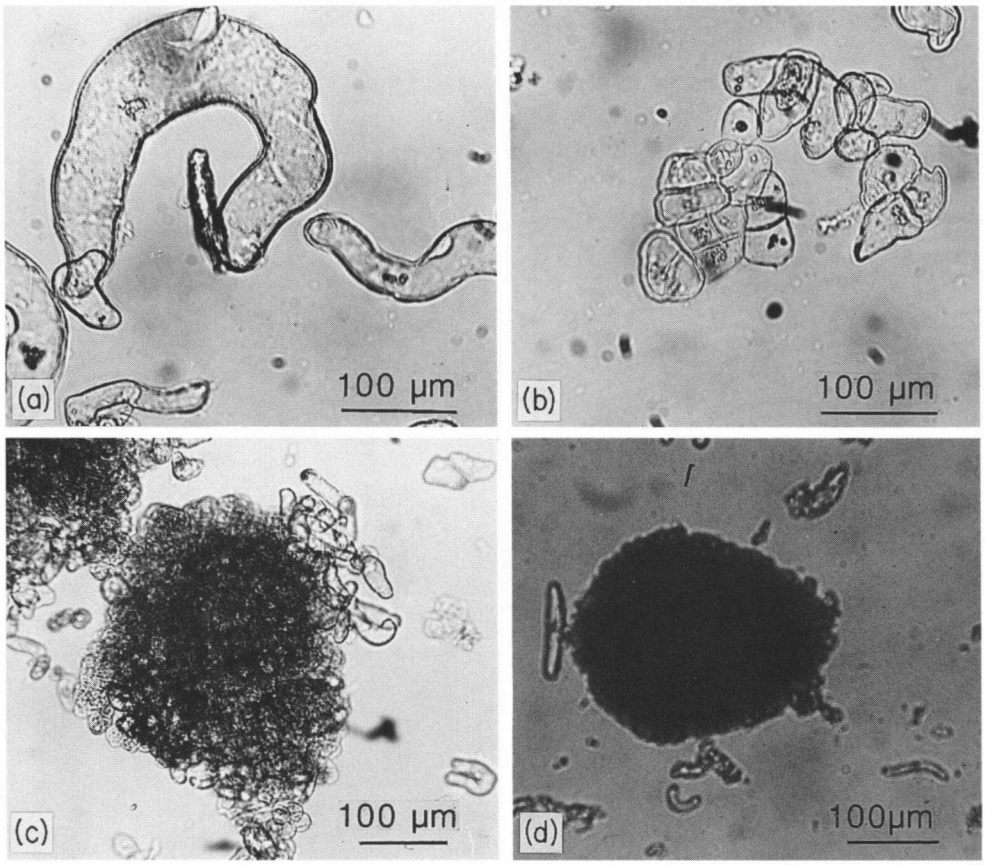

Fig. 5. Photographs of: (a) elongated cells, (b) 125-µm sieve fraction, (c) cluster of isodiametric cells, (d) globular stage somatic embryo (clusters and globules vary in diameter, both are present in the 212–330 µm sieve fraction).

embryo axis. On maturation medium they enlarge (7) and on regeneration medium they form a root, but no shoot; the shoot meristem is blocked in development. The mature polar stage (7) contains numerous vascular elements (Fig. 7b).

The mass spectrum of the ovoids (5) (Fig. 8a) is very different from that of the globules (4) (Fig. 6b): mass peaks for pentosans, ferulic acid and *p*-coumaric acid are observed. Further, mass peaks are observed for syringyl lignin and smaller amounts of guaiacyl lignin. At higher masses, mass peaks for β-sitosterol and stigmasterol are observed. Lipids are present only in very low abundances. In the mass spectrum mass peaks are observed of nucleic acids and proteins.

Single somatic embryos are produced in liquid proliferation medium and they grow and develop in maturation medium up to a polar stage of approximately 5 mm (7). During growth from ovoid (5) to polar stage of 5-mm (7) the relative abundance of mass peak *m/z* 194 increases faster than *m/z* 150 (Figs 8a and b). Also, mass peaks for lignin have increased in intensity in the 5-mm long polar somatic embryo (Fig. 8b), and in the higher mass range mass peaks for lignin dimers are observed (*m/z* 272 (G–C=C–G), 302 (G–C=C–S) and 332 (S–C=C–S) (G:guaiacyl, S: syringyl)) for stilbenes and (*m/z* 358,

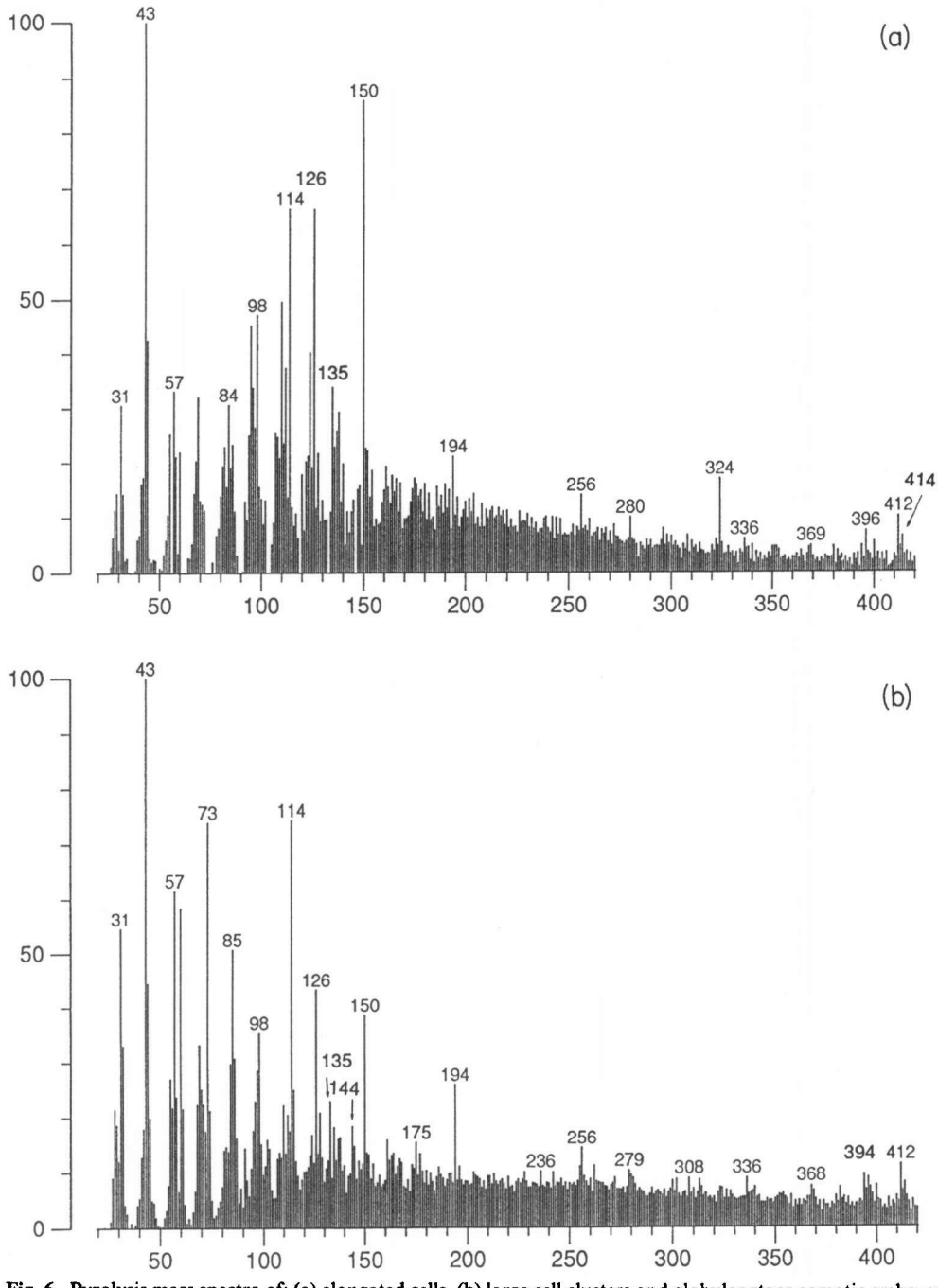

Fig. 6. Pyrolysis mass spectra of: (a) elongated cells, (b) large cell clusters and globular stage somatic embryos (300-μm sieve fraction).

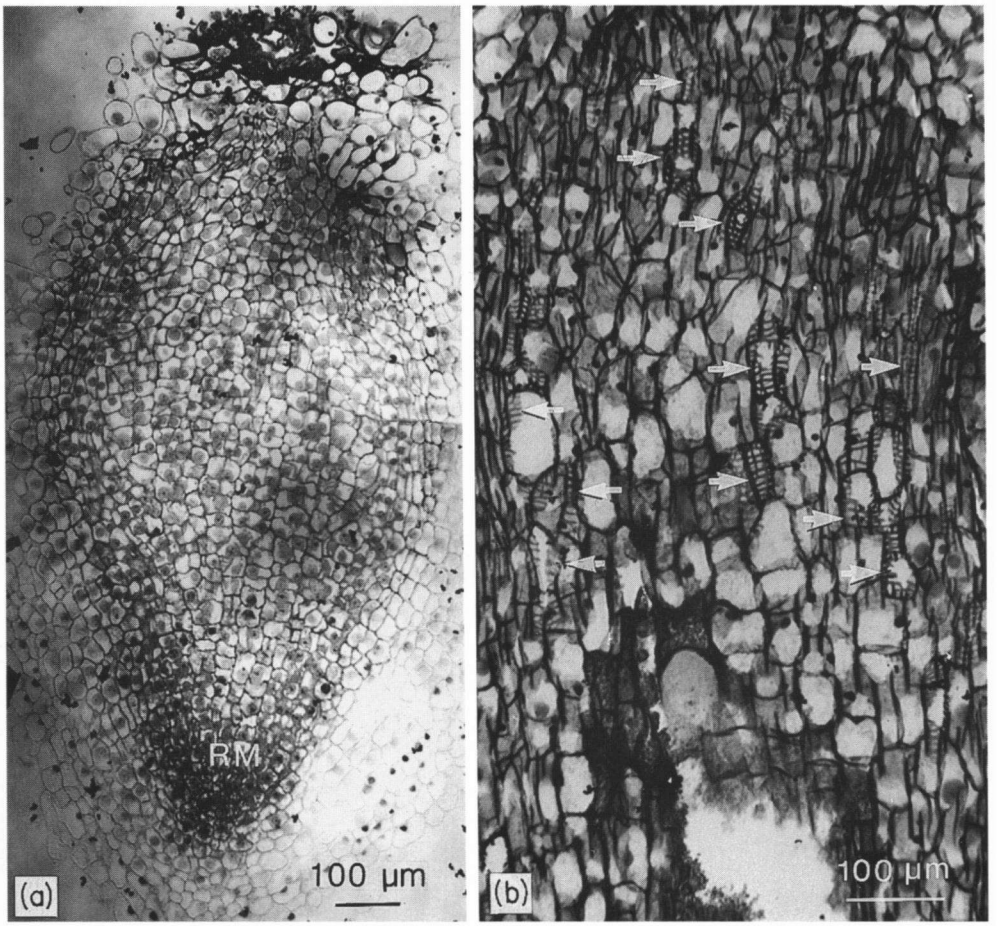

Fig. 7. Histology of: (a) ovoid stage single somatic embryo (RM=root meristem), (b) vascular cells in polar stage single somatic embryo (arrows point at vascular cells).

388, 418) for resinol type structures. The relative abundance for nucleic acid mass peaks has decreased considerably.

Developmental stages of somatic embryos attached to callus: comparison to zygotic embryos

Somatic embryos attached to callus follow a histo-differentiation pattern comparable to zygotic embryos, if matured on embryo maturation medium (Emons and Kieft 1991). After 10 days on this medium all the histological characteristics of the zygotic embryo (11) are present (Figs 9a, b) (9). Figures 9a and b are sections through a somatic embryo, grown for 9 days on embryo-maturation medium. Figure 9a, stained with toluidine blue, shows the histology of the somatic embryo. Figure 9b is another section through the same embryo stained with potassium iodide, showing starch accumulation in the scutellum.

In Fig. 10a a mass spectrum is shown of a somatic embryo (9) harvested at day 9 after transfer of embryogenic callus to embryo maturation medium. Comparison of this stage

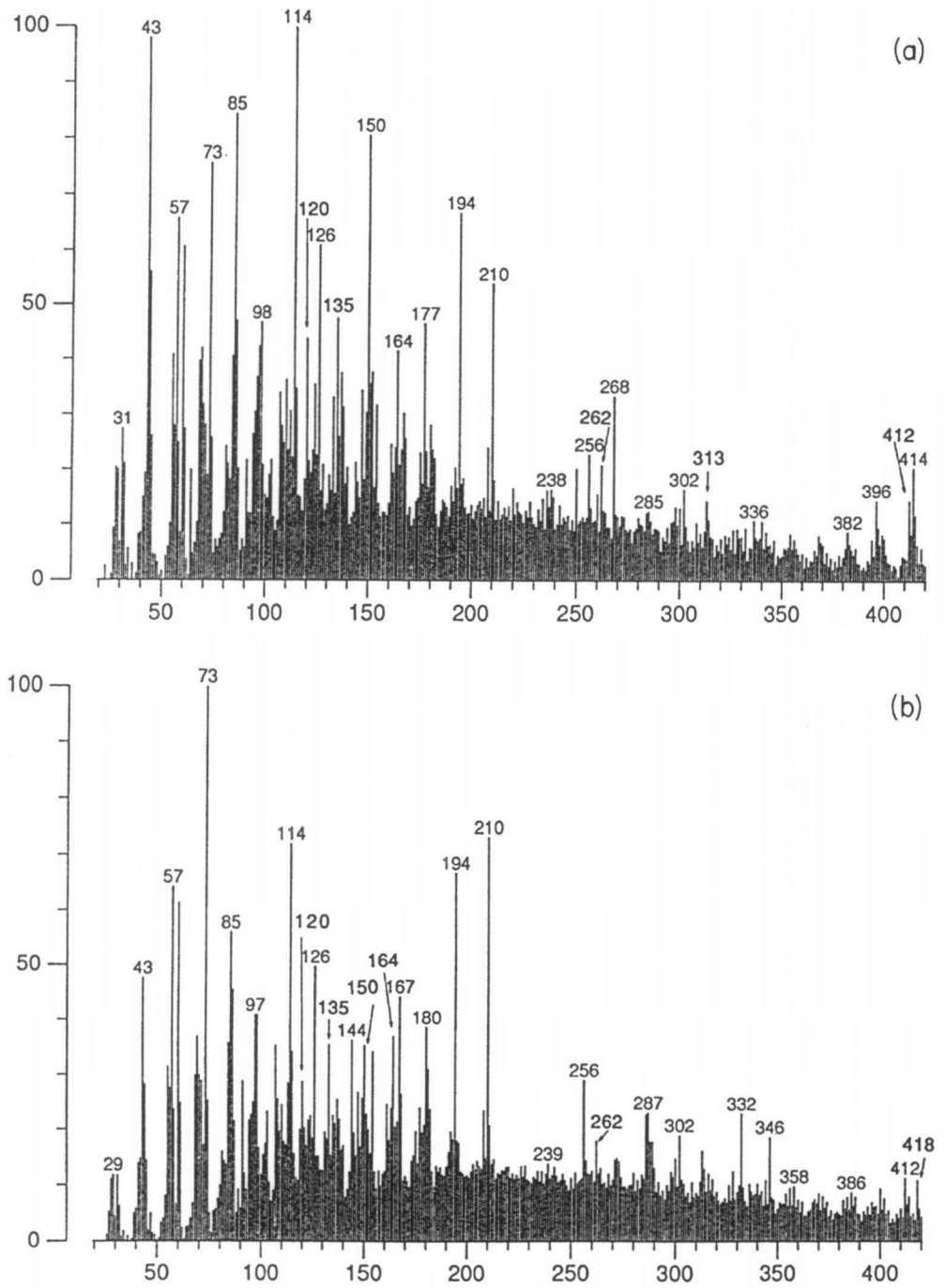

Fig. 8. Pyrolysis mass spectra of: (a) ovoid stage single somatic embryo, (b) polar stage single somatic embryo.

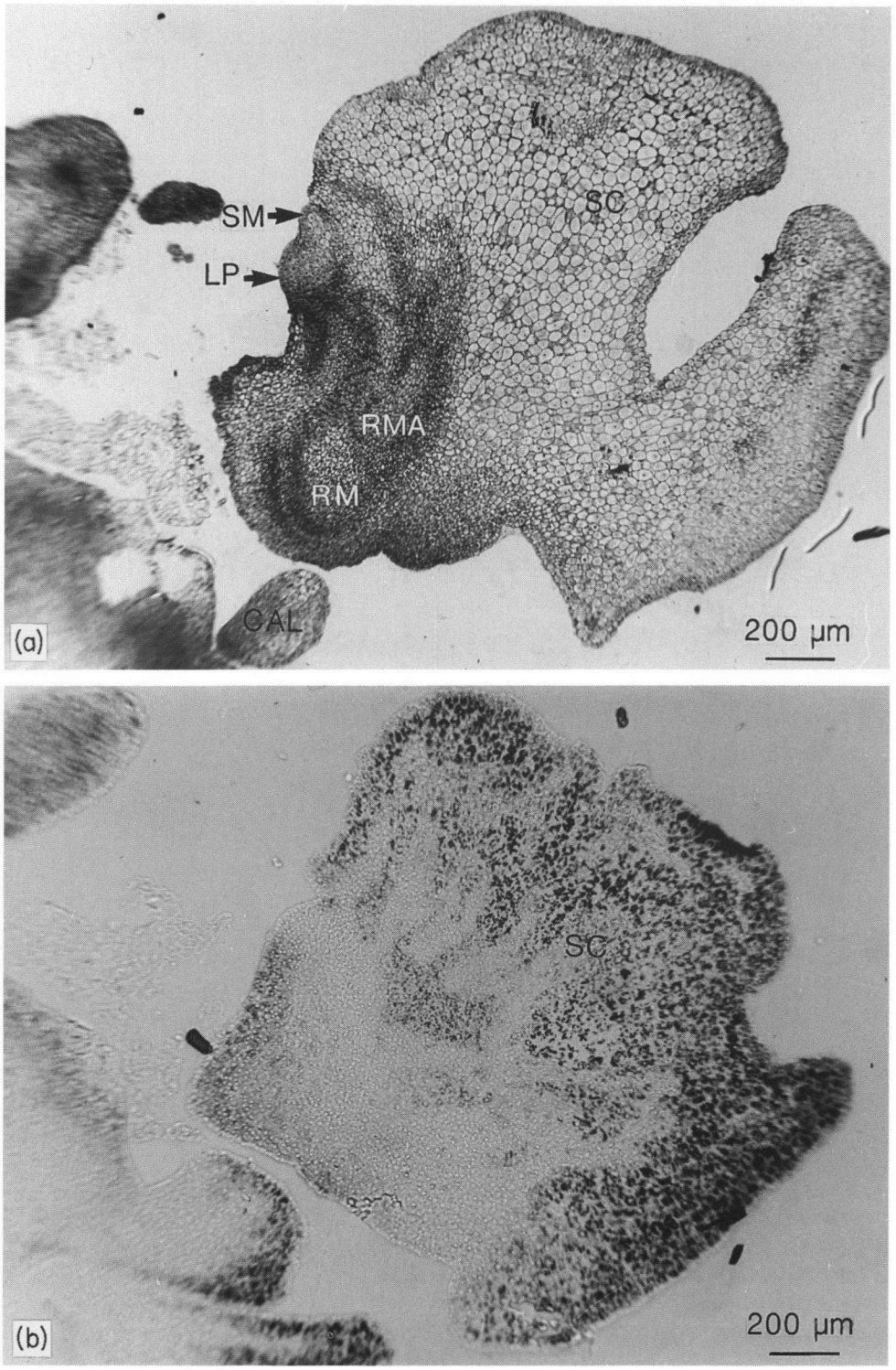

Fig. 9. (a) Histology of a somatic embryo grown for 9 days on embryo maturation medium, (b) a section through the same somatic embryo with starch grains in the scutellum cells (SC, scutellum; SM, shoot meristem; LP, leaf primordium; RM, root meristem; RMA, adventitious root meristem; CAL, callus).

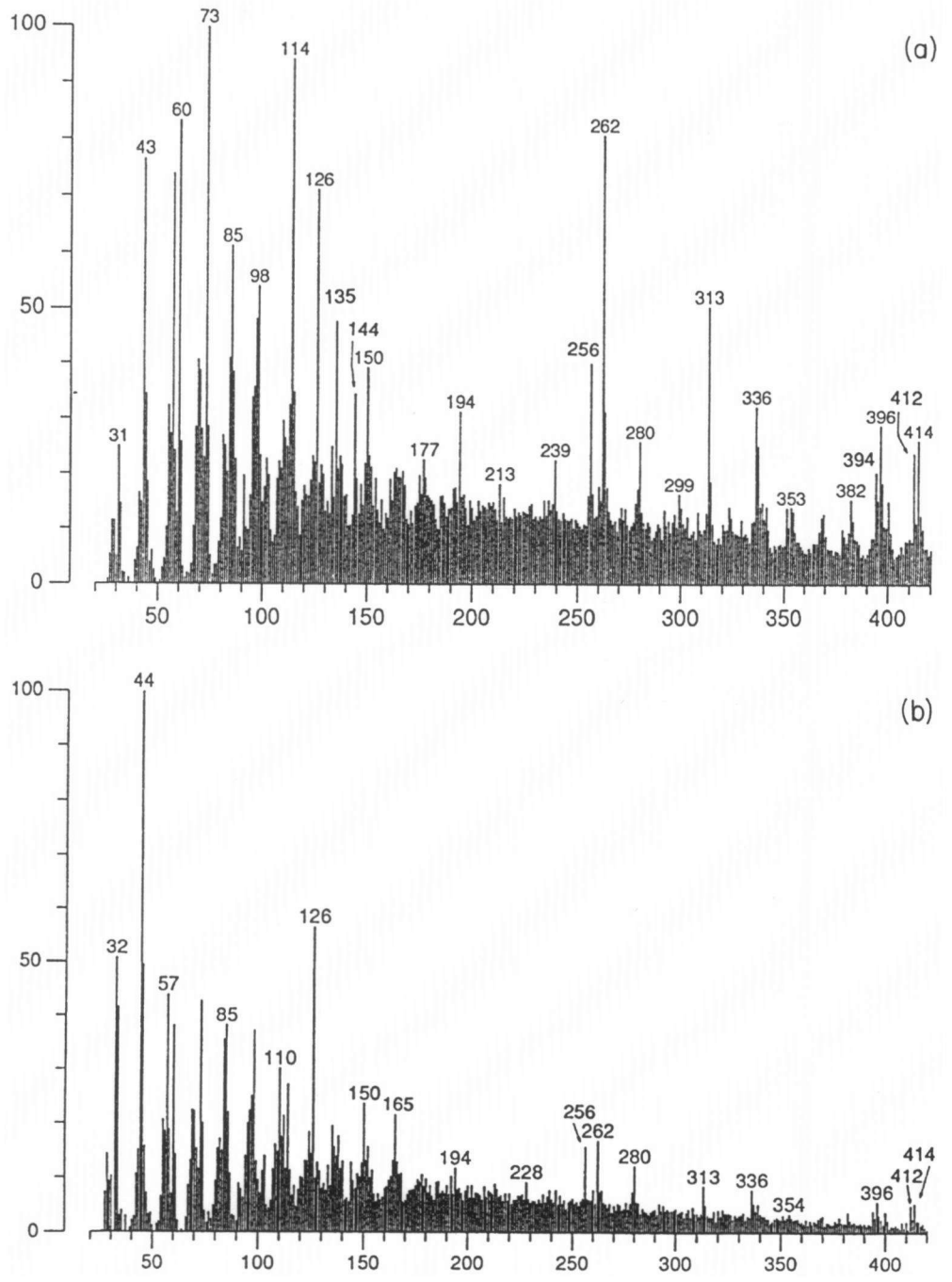

Fig. 10. Pyrolysis mass spectra of: (a) somatic embryos that had been on embryo maturation medium for 9 days (T_9), (b) somatic embryos that had been on embryo regeneration medium for 2 days, after a maturation stage of 10 days.

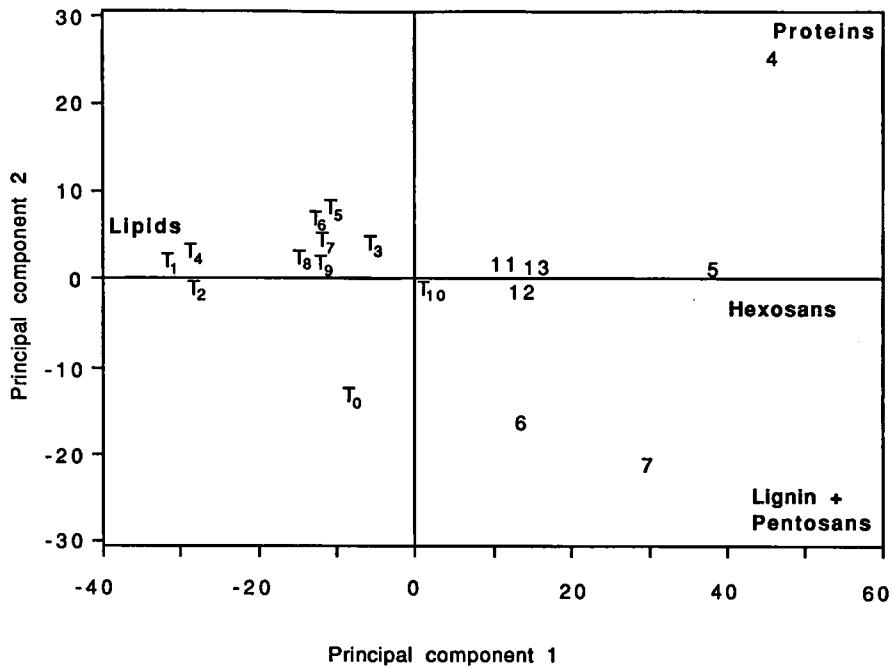

Fig. 11. Two-dimensional representation of the first two principal components; the numbers represent the various samples; T_0–T_{10}: developing stages of somatic embryos grown attached to callus; 11, 12, 13: zygotic embryos of 1–2, 2–3 and 3–4 mm length respectively; 4, 5, 6, 7: globular, ovoid, polar 3 mm-long and polar 5-mm long stages of single somatic embryos.

(T_9) with embryogenic callus (T_0) (Fig. 4) shows a relative decrease of fatty acyl groups. The relative abundances for pentosans and hexosans have increased compared to the somatic embryo of day 0 (8). The sample contains ferulic acid but no distinct mass peaks for syringyl or guaiacyl lignin are observed. After germination for 2 days (T_{10+2}) (10) (Fig. 10b), the most conspicuous feature is the decrease of fatty acyl groups. The relative abundance of proteins is lower than before germination. At *m/z* 165 a mass peak is observed of a compound which was not seen in maize embryos. Using pyrolysis gas chromatography/mass spectrometry, this mass peak could be assigned to the molecular ion of 6-methoxy-2-benzoxazolinone (data not shown).

Principal components analysis

Principal components analysis was performed on a set of mass spectra consisting of zygotic embryos of 1·5 (11), 2·5 (12) and 3·5 (13) mm length and of the two types of somatic embryos: the somatic embryos harvested on days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (8, 9) and which can develop further into plantlets, and globular (4), ovoid (5) and 3-mm (6) and 5-mm long polar stages (7) of single somatic embryos which are blocked in shoot development.

In Fig. 11 a graph is presented of the first two principal components. Principal component analysis is a multivariant analysis technique which can be used to form a linear combination of masses in order to reveal underlying chemical components by filtering the original spectra (Windig *et al.* 1981/1982). In Fig. 11 a two-dimensional

representation of this mathematical calculation is given for the first two principal components. In this plot the distribution of lipids, proteins, lignin, pentose and hexose polysaccharides is indicated. The different samples are plotted in the same graph. Each point represents a single measurement. The distance between two samples signifies the chemical difference between them. The somatic embryos attached to a callus are indicated as T_0 through T_{10} (8, 9). The number indicates the number of days on embryo maturation medium. The other samples are numbered corresponding to their number in Fig. 1.

The three stages of zygotic embryos (11, 12, 13) are characterized by high abundances of hexose polysaccharides. In contrast, the somatic embryos grown attached to callus plot on the left-hand side of the graph, characterized by high relative abundances of lipids. In general, more mature somatic embryos (T_1–T_{10}) show a tendency to plot nearer to the centre of the graph. The sample T_{10} is located at the centre of the plot. This indicates that these somatic embryos become chemically more similar to the zygotic embryos (11, 12, 13). The single somatic embryos all plot to the right-hand side of the figure. The globular stage (4) is characterized by proteins. The ovoid stage (5) is still rich in proteins but the mass spectrum also contains mass peaks for lignin. The relative abundance of lipids is lower than for the zygotic embryos and therefore this developmental stage plots further to the right. The polar stages (6 and 7) are enriched in lignin.

DISCUSSION

The accumulation of ferulic acid in cell walls of cell-suspension cultures is not unusual and was also observed in a culture of *Chenopodium rubrum* L. (Bokern *et al.* 1991). The relatively high abundance of mass peak 150 compared to 194 suggests a high degree of esterification to polysaccharides of these compounds (Wehling *et al.* 1989; Mulder *et al.* 1992; Morrison & Mulder, unpublished results). Previously, it was shown that esterified phenolic acids easily decarboxylate resulting in 4-vinylphenol (*m/z* 120) (from *p*-coumaric acid) or 4-vinylguaiacol (*m/z* 150) (from ferulic aid) (Wehling *et al.* 1989). Pyrolysis of an etherified or free acid results in the molecular ion (*m/z* 164 for *p*-coumaric acid; *m/z* 194 for ferulic acid).

Early in the development of single somatic embryos, namely at ovoid stage (5), lignin-like substances (syringyl, guaiacyl) appear before the initiation of a proper shoot meristem and a scutellum.

Also, the relative abundance of mass peak *m/z* 194 increases faster than *m/z* 150 (Figs 8a and b), which may be indicative of a higher proportion of ferulic acid, which is etherified to other phenolic compounds in lignin molecules (Wehling *et al.* 1989; Mulder *et al.* 1992). Lignin is a cell-wall bound polyphenolic constituent of cell walls of plant cells and is one of the end-products of the phenylpropanoid pathway (cf. Hagendoorn *et al.* 1990; Lewis and Yamamoto 1990). It has been suggested that the production of secondary metabolites is generally absent in most plant cell cultures and that this pathway has to be elicited (Ellis 1988). The presence of lignin compounds is one of the most obvious differences between the single somatic embryos (4–7) and the somatic embryos developing attached to a callus (8, 9).

In the single somatic embryos the relative abundance of syringyl compounds are higher than those of guaiacyl compounds. This is different from soil-grown plants, which show a higher abundance of guaiacyl compounds in stem parenchyma and

vascular bundle tissue in maize (Mulder *et al.* 1991a). Also, suspension cultures of *Glycine max* did not have the typical guaiacyl–syringyl lignins normally seen in plant angiosperms (Nimz *et al.* 1975). Similarly, in *Rosa glauca* suspension and callus cultures, a much lower methoxyl content was present than in soil-grown plants (Robert *et al.* 1989). Gaudillere & Monties (1989) have shown for maize internodes that the syringyl to guaiacyl ratio is higher in the lower internode. Thus, the single somatic embryos do not resemble the lignin type of the young tissue of the higher internodes, but that of the lower internode, which is more mature. According to the model of lignin deposition for secondary xylem cell walls proposed by Terashima (1990), syringyl alcohol units are incorporated in cell walls only after secondary cell wall synthesis has started. This would suggest that fully differentiated elements such as vascular elements are present in the somatic embryos, which is confirmed by UV microscopy.

Single somatic embryos lack a scutellum, the storage organ for the accumulation of starch. This deficiency discriminates these embryos from zygotic embryos and somatic embryos attached to a callus. The single somatic embryos follow a differentiation route for secondary metabolism leading to lignin whereas young zygotic embryos and the somatic embryos attached to a callus are not subject to this type of cell-wall differentiation and accumulate starch in scutellum cells.

A further conspicuous feature of the cultures is the occurrence of lipids in zygotic embryos and somatic embryos with normal development. These compounds represent an important food-storage material for germinating seedlings (Goodwin & Mercer 1988). These substances are easily metabolized when somatic embryos start germinating (Fig. 10b). In Fig. 3a it is observed that the mass peak for the C_{16} fragment attached to a glycerol (*m/z* 313) is higher than the $C_{18:2}$ analogue which fragments further to the fatty acyl unit. Apparently, the C_{16}-glycerol units are more stable under these ionization conditions than the $C_{18:2}$ analogue.

The presence of nucleic acids is predominantly shown in mass peaks *m/z* 111 (cytosine), 126 (thymine) and 135 (adenine). The relative abundance of guanine (*m/z* 151) is low due to the fact that this compound cannot be efficiently ionized under these conditions.

The compound 6-methoxy-2-benzoxazolinone (*m/z* 165) is not observed during embryo maturation, but is present in germinating embryos. Previously, it has been observed in young tissue of maize internodes near the meristematic area (Boon 1989). This compound and 6,7-dimethoxy-2-benzoxazolinone have been shown to possess the property of modifying the binding affinity of auxins to receptor sites in maize (Nachman 1982).

Histological investigations have shown that somatic embryos of maize on embryo maturation medium follow a development similar to that of zygotic embryos (Emons and Kieft 1991). Principal components analysis on PyMS spectra shows that normally developing somatic embryos plot nearer to the zygotic embryos in the factor-analysis graph than those blocked at the shoot side, indicating that the somatic embryos that grow attached to calli are also biochemically more similar to zygotic embryos. On the other hand, the single somatic embryos are located in the opposite corner of the same factor-analysis plot and during development they shift to a position which is determined as lignified, away from the zygotic embryos. Apparently, instead of forming a shoot meristem and accumulating starch in the first developed primordium, the scutellum, a pathway for secondary metabolism leading to lignin is followed.

ACKNOWLEDGEMENTS

We wish to thank professors M. T. M. Willemse and J. J. Boon for valuable discussions, S. Massalt for photography, and J. Pureveen and G. Eijkel for technical assistance with mass spectrometry and data handling. The mass spectrometric work is part of the research programme of Fundamental Research on Matter (FOM) with financial support from the Dutch Organization for Scientific Research (NWO).

REFERENCES

Bokern, M., Wray, V. & Strack, D. (1991): Accumulation of phenolic acid conjugates and betacyanins, and changes in the activities of enzymes involved in feruloylglucose metabolism in cell-suspension cultures of *Chenopodium rubrum* L. *Planta* **184:** 261–270.

Boon, J.J. (1989): An introduction to pyrolysis mass spectrometry of lignocellulosic material: case studies on barley straw, corn stem and *Agropyron* species. In: Chesson, A. and Ørskov, E.R. (eds): *Physico-Chemical Characterisation of Plant Residues for Industrial and Feed Use*. 25–49. Elsevier Applied Science, London, New York.

Boon, J.J. (1992): Analytical pyrolysis mass spectrometry: new vistas opened by temperature-resolved in-source PyMS. *Int. J. Mass Spectrom. Ion Process.* **118/119:** 755–787.

Chu, C.C., Wang, C.C., Sun, C.S., Hsu C., Yin, K.C., Chu, C.Y. & Bi, F.Y. (1975): Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia sin.* **18:** 659–668.

Ellis, B.E. (1988): Natural products from plant tissue culture. *Natural Product Rep.* **5:** 581–612.

Emons, A.M.C. & Kieft, H. (1991): Histological comparison of single somatic embryos of maize from suspension culture with somatic embryos attached to callus cells. *Pl. Cell Rep.* **10:** 485–488.

Emons, A.M.C. & Kieft, H. (1993): Somatic embryogenesis in maize (*Zea mays* L.). In: Bajaj, Y.P.S. (ed.): *Biotechnology in Agriculture and Forestry*. Springer Verlag (in press).

Gaudillere, M. & Monties, B. (1989): Biochemical and biosynthetic studies on lignification of gramineae. In: Lewis, N.G. and Paice, M.G. (eds): *Plant Cell Wall Polymers*. 183–192. American Chemical Society.

Goodwin, T.W. & Mercer, E.I. (1988): Lipid metabolism. In: Goodwin, T.W. and Mercer E.I. (eds): *Introduction to Plant Biochemistry*. 273–327. Pergamon Press, New York.

Hagendoorn, M.J.M., Traas, T.P. Boon J.J. & van der Plas L.H.W. (1990): Orthovanadate induced lignin production, in batch and continuous cultures of *Petunia hybrida*. *J. Pl. Physiol.* **137:** 72–80.

Lewis, N.G. & Yamamoto, E. (1990): Lignin: occurrence, biogenesis and biodegradation. *A. Rev. Pl. Physiol. Pl. molec. Biol.* **41:** 455–496.

Mórocz, S., Dudits, D. & Nemeth, J. (1986): In: Somers, D.A., Gegenbach, B.G., Biesboer, D.D., Hackett, W.P. & Green, C.E. (eds): *VI International Congress on Plant Tissue and Cell Culture*. 190. Minneapolis.

Mulder, M.M., Dolstra, O.M. & Boon, J.J. (1991a): Pyrolysis mass spectrometry as a tool in plant breeding. In: Gulletti, G.C. (ed.): *Production and Utilization of Lignocellulosics*. 291–307. Elsevier Applied Science, London, New York.

Mulder, M.M., Pureveen, J.B.M., Boon, J.J., Martinez, A.T. (1991b): An analytical pyrolysis mass spectrometric study of *Eucryphia cordifolia* wood decayed by white-rot and brown-rot fungi. *J. analyt. appl. Pyrol.* **19:** 175–192.

Mulder, M.M., van der Hage, E.R.E & Boon, J.J. (1992): Analytical *in source* pyrolytic methylation electron impact mass spectrometry of phenolic acids in biological matrices. *Phytochem. Anal.* **3:** 165–172.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Pl.* **15:** 473–497.

Nachman, R.J. (1982): Convenient preparation of 2-benzoxazolinones with 1,1-carbonyldiimidazole. *J. heterocyc. Chem.* **19:** 1545–1547.

Niemann, G.J., Pureveen, J.B.M., Eijkel, G.B., Poorter, H. and Boon, J.J. (1992): Differences in relative growth rate in 11 grasses correlate with differences in chemical compositon as determined by pyrolysis mass spectrometry. *Oecologia* **89:** 567–573.

Nimz, H.H., Ebel, J. & Grisebach, H. (1975): On the structure of lignin from soybean cell suspension cultures *Z. Naturf.* Teil C. **30,** 442–444.

Prioli, L. & Sondahl, M.R. (1989): Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.). *Biotechnology* **7:** 589–594.

Rhodes, C.A., Lowe, K.S. & Ruby, K.L. (1988): Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. *Biotechnology* **6:** 56–60.

Robert, D., Mollard, A. & Barnoud, F. (1989): ^{13}C NMR qualitative and quantitative study of lignin structure synthesized in *Rosa glauca* calluses. *Pl. Physiol. Biochem.* **27:** 297–304.

Shillito, R.D., Carswell, G.K., Johnson, C.M., DiMaio, J.J. & Harms, C.T. (1989): Regeneration of fertile plants from protoplasts of elite inbred maize. *Biotechnology* **7:** 578–581.

Terashima, N. (1990): A new mechanism for formation of a structurally ordered protolignin macromolecule in the cell wall of tree xylem. *J. Pulp Paper Sci* **16:** J150–J155.

Vasil, V. and Vasil, I.K. (1986): Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L. *J. Pl. Physiol.* **124:** 399–408.

Wehling, K., Boon, J.J., Willemse, M.T.M. & Wiermann, R. (1989): *p*-Coumaric acid— a monomer in the sporopollenin skeleton. *Planta* **179:** 376–380.

Windig, W., Kistemaker, P.G. & Haverkamp, J. (1981/1982): Chemical interpretation of differences in pyrolysis-mass spectra of simulated mixtures of biopolymers by factor analysis with graphical rotation. *J. analyt. appl. Pyrol.* **33:** 199–212.

Wood, G.W. (1980): Complex lipids. In: Waller, G.R. and Dermer, O.L. (eds): *Biochemical Applications of Mass Spectrometry.* 173–228. Wiley-Interscience Publications, New York.