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SOMATIC EMBRYOGENESIS IN ASPARAGUS OFFICINALIS L.

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SOMATIC EMBRYOGENESIS IN *ASPARAGUS OFFICINALIS* L.

By

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ABSTRACT

SOMATIC EMBRYOGENESIS IN ASPARAGUS OFFICINALIS L.

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The roles of explant source, auxin type and concentration, and carbohydrate source and level in asparagus somatic embryogensis were examined. Calli derived from in vitro established crowns (IVC) and lateral buds (LB) had a high embryogenic capacity; yielding 81-127(IVC) and 46-69 (LB) globular and bipolar mature embryos/g callus, on induction media (IM): Murashige and Skoog salts and vitamins (MS) + 2,4-D or NAA (0-0.1ppm) or kinetin at levels of 0.01-0.1ppm. Calli derived from spear sections (SS) had a lower embryogenic capacity where only 9-23 embryoids/g callus formed on IM. The auxin 2,4-D at levels of 1-10ppm induced a high embryogenic frequency. However, 2,4-D also induced formation of abnormal embryos and a low rate of embryo conversion to plantlets on maturation medium (MM) of MS + NAA (0.05ppm) and kinetin (0.1ppm). On the other hand, NAA of the same levels induced a lower embryogenic frequency. However, NAA promoted normal embryo formation and a higher conversion rate than 2,4-D; up to 2(SS), 10.7(LB) and 13.7 plantlets/g callus (IVC) within six weeks on MM.

Glucose (4-5%) in IM containing 2,4-D (1.5ppm) significantly increased embryo formation while sucrose of the same levels had an intermediate effect and fructose reduced formation. However, in IM containing NAA (1.5ppm) the effect of glucose on embryo formation was not as pronounced, and there was no significant difference between glucose and sucrose, while fructose reduced formation. Fructose in MM enhanced the rate of embryo conversion into plantlets, and the combination sucrose in IM and fructose in MM at the level of 5% gave the highest rate (10.2 plantlets/g callus). Carbohydrate levels significantly affected conversion rate where levels of 2% gave the lowest while 5% yielded the highest rate; 3.4 and 7.4 plantlets/g callus, respectively.

Embryogenic cell suspension cultures have been established and maintained over a 10 month period in liquid IM of MS + NAA (10-20ppm). Globular embryos were transferred from liquid IM to solidified MM containing glucose, fructose or sucrose (2-10%). Carbohydrate levels of 4-10% in MM enhanced the germination of somatic embryos to plantlets by 3.6-8.5 fold compared to 2%. Furthermore, transfer of the embryos from these levels to MM with a lower level (2%) enhanced the conversion of embryos into plantlets by 2-4 fold.

DEDICATION

To my dear friend, Allison Fine

and her children,

Sheba, Ben and Nani

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INTRODUCTION

Asparagus officinalis L. is a monocot species that provides interesting challenges for breeding and physiological research. Asparagus has a low multiplication rate using traditional horticultural methods (Anderson and Ellison, 1965). These methods have restricted asparagus breeding programs and the selection and propagation of elite crowns (Yang and Clore, 1975). However, in recent years, efforts have been focused towards production of selected asparagus crowns by in vitro propagation (Murashige et al. 1972; Hasegawa et al. 1973; Yang and Clore, 1973; Yang, 1977; Volokita et al. 1987).

Despite many experiments, monocots have been found much more difficult to regenerate in culture than dicots (Vasil, 1987). However, successful regeneration and preliminary work on somatic embryogenesis has been reported in asparagus (Wilmar and Hellendorn, 1968; Steward and Mapes, 1971) and in other species of the Liliaceae family (Reuther, 1983).

The goal of this work was to examine the roles of explant source, growth regulators and carbohydrate source and concentration on asparagus somatic embryogensis. Based on the information obtained in these experiments, preliminary studies on large scale production were

conducted. It is demonstrated here that somatic embryogensis in asparagus can be obtained through different explant types and media sequences and that explant source, hormonal and nutritional factors play an important role.

REVIEW OF LITERATURE

The genus Asparagus belongs to the Liliaceae family and was first described by Lineaus (1775). Asparagus is comprised of about 150-300 herbaceous and woody perennial species spread throughout the temperate and tropical regions of the world (Baily, 1942; Lawrence, 1982). Asparagus officinalis L. is the only species cultivated as an edible vegetable plant. It is an European-Sibirian continental plant and belongs to the northeast Mediterranean vegetation gene center (Reuther, 1984; Peirce, 1987). Asparagus officinalis L. is considered one of the oldest garden vegetables. The ancient Greeks introduced it to their land from the eastern part of the Mediterranean. During the Imperial Roman era, it was a popular vegetable. It was probably the Roman Legions that subsequently introduced it to central Europe. This assumption is based on the fact that wild asparagus can be found in the vicinity of the ancient Roman Legion camps (Luzny, 1979). The name asparagus probably originated from the old Iranian word "Sparaga" which means shoot, rod, spray. The Greeks used the word "Asparagos". This was converted to the common name "Asparagus" which has been used by Roman and other European nations (Luzny, 1979).

Native asparagus species are described in several regions in Europe. Henderson (1890) described it as native to Great Britain, Russia, and Poland. It is suggested that the native forms of asparagus originated from cultivated types which then reverted to wild forms (Luzny, 1979). In Great Britain, asparagus grew in most vegetable gardens (Sturtevent, 1919). From there asparagus arrived in New England with the Puritans in the early seventeenth century. Here it escaped from cultivation and became adapted to sandy fields, roadsides and garden sites. By 1776 asparagus was growing in every colony along the Atlantic coast (Gleason and Cronquist, 1963). During the following century it became widely distributed in North America. Today, it grows everywhere in the United States except in areas of extreme heat. It is also grown as a high value crop in different parts of the world. California is the main producer in the United States, and Washington is second. In Michigan, the third largest producer, asparagus is grown on sandy soils not suitable for other vegetable crops.

Properly maintained asparagus fields should remain productive for up to 20-25 years, producing annual yields of 3,000 pounds per acre (Ellison, 1986). However, despite the increase in asparagus production due to increased plantings, asparagus yields are significantly declining (Hartung, 1987). Presently asparagus fields are being removed after 8-15 years production due to decreased plant stands and concomitant decrease in spear size (Takatori and

Souther, 1978). The average yield for Michigan in 1974 was 1,600 pounds per acre; whereas, in 1981 it decreased to 900 pounds per acre. Toward 1987 it increased to 1,200 pounds per acre due to new planting (Michigan Crop Reporting Services, 1987).

Most asparagus fields are planted with approximately 10,000 crowns per acre, but often fewer than half of the original crowns survive after the first five years. A 1978 survey of asparagus fields in Michigan showed that the average crown population was only 3,153 crowns per acre. This represent a 70% reduction in crown survival. This decline in yield and number of crowns is known as the "Asparagus Decline Syndrome" and is not confined only to Michigan (Hartung, 1987). During the last four decades, asparagus production in New Jersey declined from 30,000 acres to less than 1000 acres (Herner and Vest, 1974). In California, the acreage of asparagus fields decreased from 44,000 acres in 1974 to 28,000 acres in 1978 (Takatori and Souther, 1978). Asparagus decline has also been reported in the Netherlands where it decreased from 500 ha in 1963 to 340 ha in 1970 (Vanbakel and Kerstens, 1970). In addition to the decreased longevity and productivity of established plantings, asparagus is re-established with difficulty in fields where asparagus was previously grown (Hanna, 1947; Hartung, 1987). The fusarium crown and root rot diseases are considered the main factors in the decline of asparagus fields, and no solution has yet been found (Hartung, 1987).

Botanical Description

Asparagus officinalis is winter hardy and is considered a cool season crop with 24 to 29 C day, and 13 to 19C night temperatures favoring productivity and longevity (Peirce, 1987). As a perennial grass in the lilly family, asparagus establishes a crown which is an extensive underground mass of fleshy roots, fibrous roots, and stems (rhizomes). The rhizomes give rise to spears that develop into 4 to 6 ft tall ferns (Pierce, 1987). The fleshy or tuberous roots spread laterally to an 8-12 foot depth (Shelton, 1978). Each spring new fleshy roots arise from primary and secondary roots and also adventitously from rhizome tissue (Jones and Rosu, 1928). The fleshy roots give rise to the fibrous roots which function as absorbtive organs. The fibrous roots die in late fall at the end of the growing season and new ones develop the following spring. Each rhizome carries one or more lateral buds that give rise to new spear growth. The spears are triangular and consist of short internodes and lateral buds covered with pappery bracts. The bracts are the true leaves but do not have photosynthetic function. Instead, photosynthesis is active in the cladophylls (Pierce, 1987). Structurally, a cross section of a spear shows five anatomical regions: the epidermis, cortex, pericyclic fibers, ground parenchyma, and vascular bundles (Pierce, 1987).

Elongation of spears proceeds first at the lowest internode and then within successive internodes resulting

in rapid spear growth. As the spears develop, cell walls in the pericycle and vascular bundles gradually become lignified, starting at the base of the spear (Sosa-Coronell, 1974). However, the fiber cell walls at the shoot tip region remain thin since no lignin deposition occurs there.

Asparagus officinalis L., is a dioecious species with a 1:1 sex ratio. Sometimes single plants will produce perfect flowers. This is a result of a rare mutation in the male plants which revert them to the andromoncious state. Andromoncious plants can be 10 to 20% of some asparagus populations (Wricke, 1979). The male flowers are slender, bell-shaped and greenish white in color. Each flower has an aborted ovary and well developed anther bearing orange pollen. Female flowers are smaller than those on male plants and contain vestigial, functionless anthers and a well developed ovary, style and three feathered stigma. The asparagus fruit is a red berry containing from one to six small seeds which are nearly round, flattened on one side with a hard black coat. The seeds are primarily endosperm tissue with a small elongated embryo. As the seed germinates, a radical appears first, followed by the primary stem. As subsequent shoots develop, the primary shoot senesces.

There are also important differences in the growth of female versus male plants. Females produce fewer spears that are larger in diameter than those of male plants. On the other hand, male plants produce overall a greater number of

spears and show greater longevity than females. (Ellison and Scheer, 1959). Also, male plants do not produce seedling weeds which compete with the established crowns and may harbor diseases. For these reasons there are major efforts to produce all-male varieties (Ellison 1986).

Breeding Goals

Today, fusarium is considered the most limiting factor in asparagus production and resistance to it is listed as the most important goal in asparagus breeding. Over 26 vegetable varieties with resistance to fusarium wilt have been developed through plant breeding (Mace et al. 1981). Grogan and Kimble (1959) suggested that resistant varieties are the most viable tool to arrest fusarium infection. In their selection process for developing resistant plants, they found only one asparagus plant line, which exhibited limited tolerance to fusarium. Takatori (1978) in California and Ellison (1986) in New Jersey made attempts to select for asparagus with resistance to fusarium, but to date no such cultivar has been found. Ellison (1986) also considered the possibility of using exotic germplasm in breeding for fusarium resistance. He collected numerous seed samples of A. acutifolius in the wild i.e. in Crete, mainland Greece, Italy and Spain, and seed of wild A. maritimus from Yugoslavia. Unfortunately, all of these accessions were found to be susceptible to fusarium. So far only the ornamental species A. springerii and A. plumosus have been found to be highly resistant to fusarium, but

neither species will sexually hybridize with A. officinalis (Ellison 1986).

Additional breeding goals are high yield and quality characterized by an increased number of spears of large diameter per plant, uniformity among the spears, low level of fiber, high resistance to diseases, and climatic adaptability.

Breeding Approach

As a species with male and female plants, asparagus offers interesting breeding challenges. Lack of genetic markers is also a considerable barrier in asparagus breeding. Marker gene(s) to distinguish between male and female plants still have not been found. Such a marker would be of great value, since it would enable identification of male and female plants in early stages. So far, the inheritance of only a few morphological genes has been determined in asparagus. These include those involved in chlorophyll and carotene synthesis (Irizarry et al. 1965), and the purple stalk recessive gene which is a useful marker in identification of haploid seedlings (Bassett et al. 1971). Future mapping of the asparagus genome using isozymes and DNA techniques will make it possible to find more genetic markers useful in breeding.

As a perennial grass, asparagus has a long life cycle. Asparagus plants start to yield after 4 to 5 years in the field, and the average harvesting period is about 10-15

years. Therefore, the development of new cultivars requires many years (Ellison, 1986). According to Ellison, Norton (1913) was the only one that was able to selecte for asparagus plants with resistance to disease. This may be because Norton consistently utilizing mass selection, screening large numbers of plants from different sources.

It is likely that genetic diversity among the asparagus cultivars in North America is relatively low. Since asparagus was cultivated by the Greeks more than 2,000 years ago, and introduced by the Roman Legions to central Europe, the native forms of asparagus presently found there, may have originated from these cultivated types that reverted to wild forms (Luzny, 1979). Furthermore, it was brought to North America by the colonists from discreate regions in Europe (Gleason and Cronquist, 1963). In addition, essentially every strain of asparagus developed in the United States and Canada since 1930, has been a selection of Martha or Mary Washington (Ellison, 1986). Still, there is considerable variation in yield of individual plants of the same variety. Propagation of large numbers of clones from selected crowns will enable a better evaluation of such crowns at different field plots. Rigorous determination of the genetic diversity of asparagus would be assisted by comprehensive isozyme and DNA analyses.

Tissue Culture

Asparagus has a low multiplication rate using conventional propagation methods. Only a limited number of

propagules can be obtained by division of a mature crown. Experiments in establishing rooted cuttings were of limited success (Anderson and Ellison, 1965). Yang and Clore (1973, 1975) offered an alternative method of propagating aerial crowns of asparagus stems in potted plants kept under high moisture. Following enlargement, shoots and roots are formed and are separated artifically to form new plants. However, this technique is slow and does not produce a large number of plants. So far, in vitro tissue culture techniques have proved to be the most promising for propagating asparagus lines (Hasegawa et al. 1973; Yang and Clore, 1973; Chin, 1982; Reuther, 1984). Several studies have been devoted to developing practical in-vitro techniques for vegetative mass propagation of asparagus. Complete plantlets have been successfully obtained from: 1) shoot apexes excised from young lateral branches of field-grown ferns (Murashige et al. 1972), 2) apices of terminal and lateral buds of field-grown spears (Chin, 1982; Volokita et al. 1987), and 3) shoots and crowns produced in-vitro (Hasegawa et al. 1973).

Depending on the hormonal and nutrient composition of the medium, asparagus explants may form shoots, roots or callus. Rapid formation of shoots and roots, from shoot apices excised from buds, occurred on modified MS medium containing NAA (0.3ppm) and kinetin or 2iP (0.1ppm) (Yang and Clore, 1973). Similar results were observed in other studies where auxin and cytokinin levels were varied from 0.01-0.3ppm for NAA, and from 0 - 0.1ppm for kinetin or 2iP

(Chin, personal communication; Matsubara et al. 1973; Reuther, 1984; Yang, 1977; Volokita et al. 1988). In addition, shoot tip explants (produced in-vitro) formed roots when induced with NAA (0.1ppm) followed by transfer to kinetin free MS medium (Reuther, 1984).

A two step procedure including two hormonal levels promoted root formation (Volokita et al. 1988). In the first step buds from field grown spears or shoot segments produced in-vitro were placed on induction media of MS + NAA (0.3-0.8ppm) and kinetin or 2iP (0.1-0.3ppm). In the second step shoots and buds are divided and subcultured on MS with lower levels of NAA (0.03-0.08ppm) and kinetin or 2iP (0.01-0.03ppm), or hormone free MS medium. Within 2-3 weeks a large number of crowns formed roots. Cyclical splitting and subculturing of crowns produced a large number of rooted plants. Reculture of the plants on MS medium lacking NAA was conditional for successful transfer of the plants from in-vitro culture to the planting medium (Hasegawa et al. 1973). Histological examination showed that formation of roots is initiated adventitiously in a swollen mass of a callus-like tissue at the base of the shoot apex explant; whereas, spear formation resulted from growth of the axillary buds (Hasegawa et al. 1973).

Experiments on callus formation and regeneration of adventitious shoots have been conducted using shoot tips (Reuther, 1977), apical and lateral buds of spears (Chin, 1982), spear segments (Takatori et al. 1968; Yakewa et al.

1971a, b), hypocotyls (Wilmar and Hellendoorn, 1968), and stem explants (Harada, 1973). Additionally, single cells of cladophylls (Jullien et al. 1979) were induced to form callus. Hypocotyl-derived callus formed a large number of dense globular embryo-like bodies when placed on Linsmaier and Skoog (1965) (LS) basal medium containing 4.5 uM 2,4-D and 1.5 uM kinetin followed by subculture to liquid medium of the same composition. Globular bodies (larger than 2mm) continued to dedifferentiate in suspension, and their number increased with decreased 2,4-D levels (Wilmar and Hellendoorn, 1968). Spear segments freed of lateral buds formed callus within 4-6 weeks on MS medium supplemented with NAA (0.5ppm) and 15% Coconut Water (Takatori et al. 1968). Replacement of 2,4-D by NAA (0.1-1ppm), in combination with BAP (0.1-1ppm) promoted root and shoot regeneration in calluses derived from spear segments (Yakuwa et al. 1971 a,b). Harada (1973) examined the effect of auxins, cytokinins and casein hydrolysate on formation of callus, roots and shoots from stem explants. NAA (0.1-1ppm) enhanced callus growth more than 2,4-D, while zeatin, BAP and kinetin had equal effects. Adventitious roots developed slightly more with NAA (1ppm) than with 2,4-D of the same level. Cytokinins had an inhibitory effect on rooting. This effect was highest with kinetin (0.01ppm), moderate with BAP, and lowest with zeatin. NAA (0.1ppm) in combination with zeatin (1ppm) promoted shoot regeneration from callus. BAP and kinetin were less effective when

replaced zeatin, and casein hydrolysate was stimulatory only for shoot development. Explanted shoot tips formed callus on MS medium supplemented with NAA (1ppm), and callus regenerated shoots on MS + BAP and IAA (Reuther, 1977). Shoot tip cultures often developed excessive callus which competed with the newly formed roots and frequently prevented formation of a vascular connection between the root and shoot. Incorporation of growth retardants such as ancymidol, which inhibits GA synthesis (Dennis et al. 1965), reduced the proliferation of excessive callus and promoted growth of more vigorous shoots and roots (Chin, 1982; Khunachak et al. 1987).

Somatic Embryogensis

Somatic embryogenesis is a developmental sequence linked to the totipotent ability of plant cells to express their genetic potential in a pathway similar to that of the zygotic embryo (Raghavan, 1986). Steward et al. (1958) were the first to demonstrate the totipotency of plant cells by culturing carrot callus in liquid medium where single cells become disassociated and continued to divide and differentiate to form intact plantlets upon transfer to solidified medium. Steward (1963) and Wetherell and Halperin (1963) reported that a suspension culture of carrot cells regenerated an enormous number of embryoids resembling the zygotic embryos. In the same year, Takeuchi (1963) described an embryogenic sequence of development from single cells originated from carrot root-derived callus. Similarly,

Vasil and Hildebrandt (1965) showed that single cell of a hybrid tobacco, nurtured in isolation from other cells in a defined medium, formed a completely organized plant. Backs-Husemann and Reinert (1970) also demonstrated that single cells from carrot callus cultures are evolved into embryoids; thus, reinforcing the conclusion that somatic embryos indeed have their origin in single totipotent cells.

Morphological and anatomical observations show a close resemblance at the globular, heart and torpedo stages in carrot zygotic and somatic embryos (McWilliam et al. 1974) and in other plant species (Raghavan, 1986). Biochemical and physiological studies also point to the resemblance. At the biochemical level, somatic embryos of diverse plants contain fatty acids (Pence et al. 1981a), lipids (Janick et al. 1982), anthocyanins (Pence et al. 1981b), storage proteins (Crouch, 1982) and alkaloids (Schuchmann and Wellmann, 1983) that are characteristic of maturing zygotic embryos in vivo. Another example of the similarity between these cell types is the case of seeds and somatic embryos of grapes, where dormancy can be overcomed by cold treatment and is accompanied by a decrease in endogenous ABA content (Rajasekaran et al. 1982). These observations show that at least to a certain extent, somatic embryos can serve as a model for zygotic embryo development (Raghavan, 1986).

Somatic embryogenesis consists of an induction stage, early growth, and maturation and germination (conversion to plantlet) stages. In various plant species embryogenic

growth from somatic tissue occurs in two ways: direct and indirect embryogenesis. Direct embryogenesis occurs when certain cells are predetermined for embryogenic development, needing only permissive conditions to develop into an embryo (Sharpe et al. 1980, 1982). Direct somatic embryogenesis most often occurs on excised zygotic embryos (Steward et al. 1964) and somatic embryos (Kato, 1968), or on the plant arising from them, mostly from the hypocotyl region (Homes, 1986). Indirect somatic embryogenesis occurs from cells that have already been differentiated within the explant (i.e. cells of specific meristems or mature tissues). In this case, an induction treatment is needed to foster redetermination of the differentiated cells followed by development of the induced embryogenic cells (Ammirato, 1986).

The earliest cell divisions in the embryogenically determined cells may occur in a few patterns without affecting the final embryogenic outcome (McWilliam et al. 1974). Embryogenically determined cells may differentiate singly or in groups to form embryos (Williams and Mashewaran, 1986). This capability has been demonstrated when enzyme maceration of embryonic callus gave rise to many single cells which regenerated embryoids in large numbers (Button and Botha, 1975).

In many plants the treatment where an auxin promotes cell dedifferentiation and development of densely cytoplasmic globular proembryos is considered as embryo

induction stage (Halperin, 1966; McWilliam et al. 1974; Street and Wethers, 1974; Ammirato, 1985). Following transfer to an auxin-free or reduced auxin medium, proembryos continue growth and differentiate forming mature embryos and plantlets. In different cases the process of callus growth and embryogenic induction have been separated into two stages. In alfalfa for example, kinetin-NAA medium fosters growth of unorganized callus. This callus remains unorganized when transferred to media with different levels of kinetin-NAA in combination. However, somatic embryogensis is induced when such callus is transferred to media supplemented with 2,4-D (Walker et al. 1979). Cells within the proembryos retain their embryogenic competence and rather than participating in the coordinated embryo growth may themselves become embryogenic growth centers. Such patterns occur mainly in induction media supplemented with auxin where proembryos are formed, but do not continue to develop to a mature stage (Kamada and Harada, 1979). On induction medium supplemented with auxin, embryogenic cells continue to divide forming a larger proembryo, or budding off forming additional smaller, fused or separated proembryos (Ammirato, 1986). Such formation has been observed in different plant species including carrot (Street et al. 1970; McWilliam et al. 1974; Street and Withers, 1974), celery (Al-Abta and Collin, 1980), Atropa belladonna (Konar et al. 1972b), and Panicum maximum and Pennisetum purpureum (Karlsson and Vasil, 1896). Subculture of such

proembryo populations provides continuity of the process where new embryogenic cells are continuously formed (Ammirato, 1986).

On maturation medium proembryos may develop in a sequence resembling that leading to maturation of zygotic embryos, i.e. the initiation and growth of cotyledonary primordia, the early stages of cell and tissue differentiation (usually changes in cell shape) and formation of shoot and root apices. However, in culture various events may occur prematurely, be delayed, absent or extended in time, leading to morphological and developmental abnormalities in mature somatic embryos (Halperin, 1966; Ammirato and Steward, 1971; Konar et al. 1972b; Ammirato, 1985). For instance, extended cell divisions during the proembryo stage may result in relatively large proembryos and mature embryos as compared to zygotic embryos. It can also result in cells forming new centers of embryonic growth leading to multiple or secondary embryos (Ammirato, 1986b). Somatic embryos may cluster or fuse to form twins, triple and multiple embryonic bodies that generate multiple shoots and roots during plantlet formation (Ammirato 1983b, 1986a, 1986b). Somatic embryos in different developmental stages may form additional, but smaller embryos at different loci along their axis such as at the radical hypocotyl junction or on the radical cap (Halperin 1966; Konar et al. 1972; Ammirato 1985, 1986b). Developmental changes such as extensive cell divisions and/or premature cell enlargement

at the early heart-shaped stage may result in too many cell centers leading to formation of multiple cotyledons. In cases where cell division continues in the cotyledonary ring, even after the cotyledonary primordia are initiated, fused or fasicated cotyledons may be formed and develop poorly at a later stage. On the other hand, insufficient cell division or premature vacuolation and differentiation can result in aberrant or poor cotyledon formation and development. Here, somatic embryos may appear morphologically normal, but their shoot apex may be affected and fail to develop shoots during germination (Ammirato, 1985, 1986b). Changes in the developmental sequence, i.e. cell divisions, enlargement and maturation can also result in aberrant or precocious formation of the shoot apex at the same time cotyledons are formed. Such abnormalities can arrest plant development (Ammirato, 1985). On the other hand, many somatic embryos that reach the early maturation stage may precociously germinate without the growth cessation associated with seed maturation. This early germination often leads to abnormal plantlet development (Ammirato, 1986b).

Increased evidence links somatic embryogenesis to changes in the endogenous levels of auxin(s) (Fujimura and Komamine, 1979a; Sung, 1979; Kato, 1968; Raghavan, 1986). Exogenous auxins such as 2,4-D, NAA, IBA, and IAA play a key role in inducing somatic embryogenesis (Zaerr and Mapes, 1985). Carrot cells showed sensitivity to auxin and

antiauxin, during embryo formation. In habituated callus of shamuti orange, which grows in the absence of auxin and cytokinin, the addition of even a low concentration of IAA or NAA significantly reduced or inhibited embryogenesis. Conversely, the addition of auxin biosynthesis inhibitors greatly stimulated the embryogenic process (Kochba and Spiegel-Roy, 1977b). Experiments with gamma-irradiation, which is known to inactivate endogenous auxins in plants, showed that embryogenesis is stimulated when callus is irradiated prior to subculture (Kochba and Spiegel-Roy, 1977a). Such evidence suggests that the endogenous auxin level is important during early embryogenesis, whereas continual presence of auxin in the later stage is likely to inhibit embryoid formation (Halperin and Wetherell, 1964; Sung 1979). Successful embryoid formation occurred when calluses were transferred from an auxin-enriched medium to an auxin-free medium. For example, stem and embryo explants of several members of the carrot family (Umbelliferae) had the best embryogenic response in cultures where the explant was nurtured in an auxin-enriched medium for callus induction followed by transfer of callus to an auxin-free medium (Steward et al. 1970; Ammirato, 1983a). In Macleaya cordata, isolated mesophyll cells formed callus on medium supplemented with 2,4-D and kinetin. Omission of 2,4-D from the medium or its replacement with a weaker auxin such as IAA, stimulated formation of embryogenic callus (Lang and Kohlenbach, 1975). The functional connection between

reduction in auxin level(s) and embryogenesis has also been supported by experiments in which extensive washing of callus to remove 2,4-D before transfer to maturation medium enhanced somatic embryogenesis. This indicates that 2,4-D is sometimes a potent inhibitor of somatic embryogenesis (Ammirato, 1986). However, this is not the case for other auxins. For example, continuous treatment of carrot hypocotyl segments with IAA, NAA or IBA for a 5 week period favored somatic embryogenesis to the same extent as treatments where tissues were transferred to a medium lacking auxin following a 2 week period of exposure to the same auxins (Kamada and Harada 1979). Smatic embryogenesis occurred in several experiments, but mostly in low frequency when explants were cultured on auxin-enriched medium without transfer to hormone-free medium. Sharp et al. (1980) suggested that potential embryonic cells of the callus are mitotically quiescent in the presence of high auxin concentrations in the medium. A low frequency of somatic embryos may form on auxin-enriched medium. This may be due to a lack of auxin in the cellular vicinities of predetermined embryonic cells, thus enabling them to overcome mitotic arrest and form embryos. This also may explain why high embryogenic frequency occurs on the surface of fresh, white friable callus that emerges from older brownish tissues (Raghavan, 1986).

Cytokinins have been incorporated in various culture media to promote somatic embryogenesis (Zaerr and Mapes,

1985; Minocha 1987). Compounds with cytokinin activity which may exercise a considerable regulary role in somatic embryogenesis have been identified in the medium of carrot cell cultures (Salem et al. 1979). Cytokinins promote embryogenesis by enhancing division of proembryogenic cell masses. Sung et al. (1979) demonstrated a promotive role for cytokinins in somatic embryogenesis of wild-type carrots cells grown in 2,4-D enriched medium, and in 5-methyltryptophan-resistant carrot cell lines in which embryogenesis was induced by high levels of endogenous IAA. In another example (Fujimura and Komamine 1980a), the capacity of cell clusters to undergo embryogenesis was promoted by zeatin which enhanced cell division. Results leading to the same conclusion were also obtained when 2iP or isopentenyladenosine were added to embryogenic cultures of Pimpinella anisum (Ernst and Oesterhelt, 1984). In celery cell suspensions kinetin enhanced the transition of embryo from the globular to torpedo shaped stage (Al-Abta and Collin, 1978).

Despite many experiments, there has been less success with somatic embryogenesis in monocots than in dicots. Although, somatic embryogenesis has been reported in Asparagus officinalis (Wilmar and Hellendoorn, 1968; Steward and Mapes, 1971) and in other species of the Liliaceae such as Bellevilia romana (Lupi et al.1985), as well Gasteria verrucosa, and Haweorthia fascata (Beyl and Sharma, 1983) and in species of taxonomically related families, such as

Iris (Reuther, 1977) and palms (Tisserat,1979; Reynolds and Murashige, 1979). Successful somatic embryogenesis has also been reported in various species of the Graminea (Vasil and Vasil, 1982). Frequently, the embryogenic cultures are initiated from excised immature zygotic embryos (Botti and Vasil, 1983; Vasil et al. 1985; Hakman and Fowke, 1987), but other plant tissues, most notably young leaves, have also been used (Lu and Vasil, 1981; Yeh and Chang, 1986; Szabados et al. 1987). Here, the slow regeneration of callus and the rapid loss of embryonic competence are two limiting characteristics (Raghavan, 1986).

The development of monocot zygotic embryos proceeds through a well-defined pathway (Raghavan, 1972). The first division of the zygote cell gives rise to an apical cell and a basal cell. In the next round, the apical cell and the basal cell divide longitudinally and transversely, respectively. The basal cell gives rise to a suspensor complex of about 6-10 cells terminated by a large basal cell at the micropylar end and by the embryo proper at the chalazal end. Throgh active divisions the apical cell gives rise, to the spherical embryonal part, characterized by small, dense cytoplasmic cells. Compared to cells of the spherical embryo, those of the suspensor are more vacuolated and contain more endoplasmic reticulum, but appear to be depleted of ribosomes and stain less intensely for proteins and nucleic acids. This early developmental stage involves the same steps in monocots and dicots and is defined as the

globular or pre-embryo stage (Raghavan, 1976). However, there is disagreement as to whether to classify the early transverse and longitudinal cell divisions as the proembryo stage and the later formation of the spherical part as the globular stage (Raghavan, 1976, 1986). At this stage the globular zygote increases in size, mainly through active cell divisions, but retains its spherical shape. During this stage, the three primary meristems (protoderm, ground meristem and procambium) become visible. At the end of this stage, a ring of actively dividing cells appears at the apical end. Further cell divisions give rise to two portions: the lower part of the cotyledon, and to the upper part of the cotyledon and the hypocotyl, while the shoot tip is initiated at the junction of the hypocotyl and the cotyledon. A characteristic unique to the monocot embryo is the simultaneous growth of the cotyledon initial cells and the shoot apex. This may be explained by the ontogeny of monocots, in which the single cotyledon incorporated the primordia of both cotyledons and the original shoot apex found in the dicots, while a new functional shoot apex arises laterally from the subterminal tier of the proembryo. In the following stage, the process of cell differentiation in the cotyledon and hypocotyl are accompanied by considerable cell elongation resulting in formation of an elongated embryo axis. The cells derived from one half of the original basal cell form the root cap in the mature embryo, the other half of the original basal cell forms the

remaining part of the root cap and suspensor. By the time the embryo has matured, its length has increased to more than 2mm. This tremendous increase in size is supported by the uptake of raw materials from the endosperm and their utilization in the synthesis of complex substrates. As the embryo reaches maturity, the size of the individual cells is reduced to a size that is no bigger than a quarter of the early zygote cells. Mature embryos have a procambial system which differentiates throughout the hypocotyl and cotyledons, giving rise to xylem and phloem elements (Bisalputra and Esau, 1964). The mature embryo is surrounded by a mass of cellular endosperm. With the formation of a mature seed the embryo becomes quiescent with cessation in mitotic division, accompanied by a decrease in water content. The imbibition of water initiates germination which includes resumption of mitotic activity along with cell expansion and differentiation (Raghavan, 1986).

As a carbon and energy source carbohydrates (CHO) are major and essential constituents of any tissue culture medium (Thorpe, 1982). Sucrose is the sugar of transport in most plants, and is considered the best sugar for supporting growth and differentiation of in vitro plant tissue cultures, including somatic embryogenesis (Thompson and Thorpe, 1987). The constituent hexoses of sucrose, i.e. glucose and fructose and several other monosaccharides such as galactose (Gross et al. 1981) and mannose (Wright and

Northcote, 1972), disaccharides like cellobiose and trehalose (Mathes et al., 1973), lactose (Hess et al., 1979) and melibiose (Nickell and Maretzki, 1970), and trisaccharides such as raffinose (Wright and Nortcote, 1972) also can support growth in plant tissue cultures (Maretzki et al., 1974; Opekarova and Kotyk, 1973) and embryogenesis (Verma and Dougall, 1977).

Sugar alcohols such as myo-inositol, are required in small quantities for the culture of many plant species (Murashige and Skoog, 1962), but they generally do not serve as a sole carbon source. However, in a few studies sugar alcohols did support growth in vitro. Mannitol supported shoot initiation from black and white spruce epicotyl explants (Rumary, 1981) and sorbitol supported growth of malus tissue cultures (Chong and Taper, 1974). Sorbitol is considered important in the metabolism of apple and related species in vivo. The utilization of sorbitol and other cyclitols involves their conversion to the corresponding hexoses. Sorbitol dehydrogenase, which converts sorbitol to fructose, has been detected and characterized in apple callus tissue (Negm and Loescher, 1979).

Carbohydrate metabolism in vitro has been studied in various tissue culture systems. An early study (Goris, 1954) showed that carrot callus readily interconverted glucose and fructose, and synthesized sucrose. Recent studies (Komamine et al. 1978) confirmed that glucose and fructose are the most commonly metabolized hexoses, and to a

large extent initiation with different carbon sources will lead to their formation or their derived phosphates or nucleotides. The capacity of tissues to utilize different carbohydrates may vary with the species or explant (Hildebrandt and Riker, 1949) and with different cultivars (Chong and Taper, 1972). Furthermore, it may even vary with different organs of the same plant (Mathes et al. 1973). Although it has not been comprehensively studied, CHOs type may also influence the type of organ differentiated by the primary explant (Kikuta and Okazwa, 1984). Mannitol, sorbitol and inositol supported shoot bud formation in potato tuber tissue, whereas, sucrose promoted root formation.

Somatic embryogenesis may occur in two distinct stages as proembryogenic cell initiation on an auxin containing medium followed by embryo development on an auxin-free medium. It is possible that CHOs requirements and metabolism may differ in these stages. Few studies have examined the role of CHOs source in somatic embryogenesis. Galactose and galactose-containing saccharides were found to stimulate embryogenesis in habituated shamuti orange callus. Sucrose in combination with these sugars suppressed it (Kochba et al., 1978). Non-embryogenic calli lines responded to galactose at 7-10%. On the other hand, embryogenic lines responded to low galactose levels, (0.05%), which were unable to support callus growth. Here, the action of galactose or galactose containing sugars may involve

inhibition of auxin synthesis in the habituated calli (Kochba et al., 1974). Such a phenomenon was also found in other systems (Thompson and Thorpe, 1987). In Daucus carota maturation of somatic embryos on sucrose was slower than with other sugars (Verma and Dougall, 1977). In Theobroma cacao low sucrose levels (1-2%), glucose and fructose (3-5%), all stimulated faster growth than that of the zygotic embryo controls (Kononowicz and Janick, 1984). Embryogenic carrot cells on an auxin-containing medium are rich in starch (Street and Withers, 1974) which disappears during embryo formation on an auxin-free medium. It is not clear whether the metabolic changes are a direct result of the release of auxin from the medium or are associated with metabolic events involved in embryogenesis (Thorpe, 1980). Changes in starch content of organ-forming tissues have also been observed in several plant species (Brossard, 1977; Thorpe and Meier, 1974). In shoot-forming tobacco callus, maximum starch accumulation occurred just prior to meristemoid formation (Thorpe and Meier, 1972; Thorpe and Murashige, 1968). During meristemoid formation, starch was rapidly degraded (Thorpe and Meier, 1974). In meristematic tissue of cultured cotyledons of Pinus radiata (Patel and Thorpe, 1984), the decline in starch content was consistent with increased respiration and high succinate activity (Biondi and Thorpe, 1982). Higher respiration rates were also observed during shoot initiation from tobacco callus (Ross and Thorpe, 1973; Thorpe and Meier, 1972). Tobacco

callus showed increased activities of both glycolysis and the pentose phosphate pathways (Thorpe and Laishley, 1973) and increased levels of NADPH and NADP+ (Brown and Thorpe, 1980). Here, total adenosine phosphates increased during the early culture period and declined thereafter. However, the levels in shoot-forming tissue were always higher than in proliferating callus. Embryogenic cells have high mitotic activity (Dmitrieva and Mohamed, 1977; Warren and Flower, 1978), they are rich in mitochondria and have an intensified respiration rate (Street and withers, 1974).

Dissolved O2 concentration influenced embryo development in carrot (Kessell et al., 1977). Below a critical level embryogenesis occurred, but above that level rhizogenesis was favored. Lowered O2 levels increased cyanide-sensitivity and cellular levels of ATP. Exogenous adenosine at higher O2 concentrations also raised the cellular levels of ATP. Both treatments enhanced somatic embryo development (Kessell et al., 1977). These findings confirm the need for a higher energy requirement as well as for reducing power (NADPH and pentoses) for biosynthetic processes involved in organogenesis and embryogenesis (Brown and Thorpe, 1980).

The typical tissue culture medium (e.g. Murashige and Skoog with 3% sucrose) has a water potential of -5 bars (Doley and Leyton, 1970). However, under in-vitro culture conditions, the cells are exposed to water potentials similar to those of field grown plants. In soil, water

potential may range from zero, after irrigation to -15 bars at drought (Thorpe, 1982). Osmotic potential is regulated in plant cells by both inorganic ions and organic molecules (including organic acids, sugars and sugar alcohols) (Zimmerman, 1978). It has been suggested (Wyn Jones et al. 1977) that the cytoplasmic inorganic ion concentration remains fairly constant, while osmotic adaptation of the cytoplasm is mainly achieved by the accumulation of non-toxic organic molecules (Wyn Jones et al. 1977). CHOs in plant culture medium are generally in excess of the tissue growth requirement (Thorpe, 1982). In some systems, relatively large quantities of CHOs may accumulate and increase the osmotic potential in the cell, unless they are rapidly removed by metabolic utilization (Thorpe, 1982). The osmotic potential of the medium has a prominent role in growth and differentiation. An increase in medium water potential from near zero to -1 bars significantly reduced the growth rate of callus derived from wound injury of Fraxina excelsior (Doley and Leyton, 1970). At near zero water potential the callus had active surface growth and little internal differentiation. At water potentials greater than -1 bars, the callus had suberized surfaces and contained lignified xylem and scleroids. On medium with near zero water potential, callus appeared after 2-3 days, and at water potential of -6 to -10 bars it appeared after 6-9 days. Studies on the effect of mild and severe osmotic stress on tobacco tissue in culture (Klenovska, 1973) showed

that increasing the osmotic potential of the medium by 0.5 bars, with the addition of PEG 1000 at 1% w/v, reduced tobacco callus growth. The sucrose, fructose and glucose content of the tissue was also decreased. Water content of the callus was not affected but dry matter increased. More severe stress intensified these effects, and reduced the water content of the tissue. Reduction in the relative humidity of the air above the cultures from 90 to 32% increased the osmotic potential, dry weight, and sugar content of the tissue (Klenovska, 1976). An increase in medium water potential in soybean culture decreased cell size, but increased callus growth (Robarts et al. 1982). Small increases in osmotic stress (-2 bars) decreased growth of sugar cane cells in culture, increased respiration, turnover of amino acids and sucrose content. At the same time it lowered the reduced sugar content and invertase activity. These effects were even greater in cells not preconditioned to the greater medium water potential (Thorpe and Meier, 1973).

CHOs are utilized as the energy source for cell metabolism and for the formation of major cell components. The balance between these two functions varies with the stage of development (Maretzki et al. 1974). High sucrose levels promoted in vitro somatic embryogenesis (Ammirato and Steward, 1971), and development of zygotic embryos in vitro (Norstog, 1961). The younger the zygotic embryos, the higher the sugar level that was required for development in-vitro

(Yeung et al. 1981). Higher sucrose levels were also required for in-vitro rooting from cuttings of Pinus lambertiana embryos (Greenwood and Cockerline, 1978), and for root formation from sugar cane cells (Maretzki and Hiraki, 1980). On the other hand, high sucrose levels reduced embryo growth, slowed their maturation and prevented precocious germination (Ammirato, 1983). Low sucrose levels, glucose and fructose stimulated somatic embryos of Theobroma cacao to grow faster than the zygotic embryo control (Kononowicz, 1984). Increased sucrose levels reduced embryo growth. Instead, embryos produced storage lipids, anthocyanins and alkaloids characteristic of the maturing zygotic embryos. High sucrose levels affected fatty acid composition and anthocyanin content of somatic embryos comparable to the zygotic embryos (Pence et al. 1981a; Pence et al. 1981b). These findings indicated that sucrose at high levels suppressed precocious germination and regulated the embryo developmental maturation pattern (Pence et al. 1981b). Osmotic substitutes applied alone or in combination with sucrose could not duplicate the sucrose effect on regeneration of roots from cuttings of Pinus lambertiana embryos. Iso-osmotic quantities of fructose, fructose plus glucose, or glucose alone were much less effective or totally ineffective in inducing maturation of Theobroma cacao somatic embryos (Kononowicz and Janick, 1984). These findings indicated that the effect of sucrose on embryo

development is not exclusively osmotic (Thompson and Thorpe, 1987).

The optimum sucrose concentration for growth and formation of shoots in dark grown tobacco callus is 3% (Brown et al. 1979). Lower or higher sucrose levels reduced the number of shoots formed. However, the same number of shoots formed on medium with 2% sucrose when supplemented with mannitol to give the medium water potential equivalent to 3% sucrose. However, mannitol could not substitute to levels below 2% sucrose. Mannitol alone at any level could not serve as a sole energy source. Also, increased levels of bacto-agar in the medium did not replace CHOs including mannitol in promoting shoot formation. This indicated that in addition to a metabolic role, part of the CHO in the medium must be an osmoregulatory effect and that the osmoticum must enter the tissue (Brown et al. 1979).

Studies with different tobacco callus lines indicated that the requirement for optimal sugar level in the medium differed for growth, greening and shoot formation (Barg and Umiel, 1977). Reduction in sucrose level while maintaining the medium water potential by adding manitol increased the number of shoots formed and improved their morphology. During shoot formation, tobacco callus had higher water, osmotic and pressure potentials when maintained by CHO and other metabolites (Maretzki and Hiraki, 1980). As in somatic embryogenesis, bud formation from callus occurred on an auxin-containing medium and further development of shoots

occurred, following transfer of buds to an auxin free medium for (Thorpe, 1977; 1980). Reduction in sucrose level in radiata pine from 3 to 2% (Aitken et al. 1981), and in alfalfa, from 3 to 1% (Stavarek et al. 1980) promoted shoot development. Similarly, reduction by half of the mineral content of the medium and lowered sucrose levels were optimal for rooting of conifer adventitious shoots (Thorpe, 1977). The osmotic potential has a distinct effect on membrane properties, and membrane proteins such as ATPase (Zimmermann, 1978). Increased osmotic pressure intensified mitochondria activity (Thorpe, 1983) which is required for enhanced energy production during shoot initiation (Brown and Thorpe, 1980). The amplification of overall metabolic activities is also accompanied with alteration in pathway activity (Thorpe, 1978). This indicates that the changes in CHO metabolism play an integral role in differentiation. So far, detailed studies of this nature have not been conducted on somatic embryogenesis .

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SECTION I

SOMATIC EMBRYOGENSIS IN *ASPARAGUS OFFICINALIS* L.: THE ROLE OF EXPLANTS AND GROWTH REGULATORS

ABSTRACT

The role of explants and growth regulators in somatic embryogensis in asparagus was examined. Calli derived from spear-cross sections (SS), lateral buds (LB) of spears, and callus taken from crowns established in vitro (IVC) of asparagus male line (C-3) were subcultured twice at four weeks intervals on initiation media of Murashige and Skoog salts and vitamins medium (MS) + NAA (0.1-1ppm) and kinetin(0.1ppm). At the end of the second subculture calli were transferred to MS containing 2,4-D or NAA and kinetin alone and in combination at levels of 0-10ppm; designated as embryo induction media (IM). After four weeks, callus was visually and quantitatively evaluated for growth and formation of somatic embryos. The calli were transferred to maturation media (MM) of MS + (ppm): NAA (0.05) and kinetin (0.1). After six weeks on MM, somatic embryogensis was evaluated again. Calli derived from LB and IVC were found to have a high embryogenic capacity; yielding 46-69 (LB) and 81-127(IVC) embryos (small globular and bipolar mature)/g callus on IM devoid of growth regulators or with auxin and/or kinetin at levels of 0.01-0.1ppm. Calli derived from SS had a lower embryogenic capacity where only 9-23 embryoids/g callus formed on such IM. The auxin 2,4-D at levels of 1-10ppm was found to induce a high embryogenic

frequency on IM; up to 491(SS), 450(LB) and 467(IVC) small globular embryos/g callus. However, 2,4-D also induced formation of abnormal embryos and a subsequent low rate of embryo conversion to plantlets on MM; up to 2(SS), 3.7(LB) and 7.7/g callus (IVC); 2, 5.1 and 6.8%, respectively. NAA at the same levels induced a lower embryogenic frequency; up to 95(SS), 306(LB), and 251(IVC) small globular embryos/g callus on IM. However, NAA promoted normal embryo formation and a higher conversion rate than 2,4-D; up to 2(SS), 10.7(LB) and 13.7/g callus (IVC); up to 5, 21 and 17.3%, respectively. There was no difference between kinetin levels (0-1ppm) on callus growth and embryogensis. A high kinetin level (10ppm) in IM was found inhibitory to embryogenesis.

INTRODUCTION

Asparagus officinalis L. is a dioecious crop species that offers interesting challenges for breeding and tissue culture research. Asparagus has a low multiplication rate using conventional propagation methods (Ellison, 1986), where only a few propagules can be obtained by crown division (Yang and Clore, 1973). This restricts asparagus breeding where the only method for determining yield potential is through individual plant records (Hanna, 1947). So far, in vitro tissue culture techniques have proved useful for propagation of asparagus lines (Reuther, 1983). However, the vast majority of tissue culture research in asparagus has concentrated on shoot proliferation and

plantlet division from cultured meristems and shoot tips (Murashige et al. 1972; Hasegawa et al. 1973; Chin 1982; Volokita et al. 1987).

Somatic embryogenesis has been considered a potential system for propagating elite asparagus crowns (Reuther, 1983). Wilmar and Hellendoorn (1968) reported that hypocotyl-derived callus formed a large number of dense, globular embryo-like bodies when placed on Linsmaier and Skoog (1965) basal medium (LS) + 2,4-D (1.0ppm) and kinetin (0.3ppm). Globular embryoids continuously differentiated in liquid suspension of the same composition and gave rise to mature plantlets. Steward and Mapes (1971) reported the formation of somatic embryos from asparagus stem segments through a sequence of callus induction in White's liquid medium supplemented with coconut milk and NAA. At the following stage the NAA was replaced by 2,4-D. Embryos were induced in high salt medium supplemented with NAA,and root and shoot primordia were formed by transfer of embryos to medium supplemented with coconut milk and IAA. Reuther (1977) demonstrated that shoot tip- and shoot segment-derived callus formed on LS + NAA (1.0ppm) and kinetin (1.0ppm), gave rise to somatic embryos when transferred to LS + IAA (1.0ppm) and BA (0.1ppm) and subsequently to plantlets when transferred to LS or LS + IAA (0.5ppm). However, these reports did not consider the role of explant source and auxin type and concentration and did not quantify embryos and mature plantlets formed in culture.

The objectives of this study were to define an efficient scheme for producing somatic embryos of asparagus, concentrating on the role of explants and auxin type (2,4-D versus NAA). This research provides evidence that somatic embryogenesis in asparagus may occur in several pathways using different explants and media conditions, some of which were found more efficient than others.

MATERIAL AND METHODS

Spear segments (SS), lateral buds (LB), and in vitro maintained crowns (IVC) of asparagus male line C-3 were assessed for the initiation of embryogenic callus. The SS and LB explants were obtained from greenhouse grown plants, sampling the upper 2-12 internodes, from the tip of 4-7 days after spears emerge. Spears were surface sterilized for 30 min in aqueous sodium hypochlorite (1.5% v:v) and 2 drops of Tween-20, and were thoroughly rinsed with 3 changes of sterile distilled water. The SS were transverse X-sections (3-5mm) and were free of lateral buds. The LB were free of leaflets. In order to initiate callus, SS were placed on Murashige and Skoog (1962) salts and vitamins medium (MS) solidified with 0.9% Difco agar and containing 3% sucrose and supplemented with 2,4-D and/or NAA and kinetin alone and in combination at levels of 0-10ppm. The cultures were kept in the dark at 27C. In 4-8 weeks, callus was separated from SS and subcultured. Callus initiation from LB explants included two steps: 1) culture of LB for 4 weeks on MS + NAA (0.5ppm) and kinetin (0.1ppm), and 2) transfer of

LB to MS + NAA (0.1ppm) and kinetin (0.01ppm) where callus proliferated and was subcultured. IVC were maintained on MS + NAA and kinetin alone and in combination at levels of 0-1ppm under 16h/day of light 32uEm^{-2} sec^{-1} from cool white fluorescent bulbs, and gave rise spontaneously to calli. Such calli on MS + NAA (0.01, 0.1, 0.3 and 1ppm) and kinetin (0.01ppm) were separated from the IVC and subcultured. After two subcultures at 4 week intervals, calli derived from all explants were visually evaluated for growth and embryogenic capacity i.e. callus color, formation and frequency of spherical and bipolar embryos using a dissecting microscope. This information was used to determine the optimum medium for initiation of callus from each explant. For LB and IVC these were calli derived on MS + NAA (0.1ppm) and kinetin (0.01ppm), and for SS calli on MS + NAA (0.1-1ppm) and kinetin (0.01ppm). These chosen calli were all transferred to MS + 2,4-D or NAA and kinetin alone and in combination at levels of 0-10ppm, designated embryo induction media (IM). Each callus piece placed on IM was 0.4-0.6 g and the experimental unit was 3 pieces per 100 x 15 mm Petri dish. After four weeks on IM, callus increase (%) was calculated from the weights as follows: (final fresh weight - initial fresh weight) x 100 / initial weight. Somatic embryogenesis on IM was evaluated by weighing callus pieces withdrawn at random and counting the number of small globular embryos (<3mm diam), and large elongated embryos (4-7mm diam) with the latter classified as bipolar embryos.

After four weeks on IM, calli selected at random were transferred to maturation media (MM) of MS + NAA (0.05ppm) and kinetin (0.1ppm). These cultures were incubated at 27C under 16h/day irradiance 32uEm^{-2} sec^{-1} from cool white fluorescent bulbs. After six weeks on MM, evaluation of somatic embryogenesis was performed by weighing callus pieces and counting globular, bipolar, abnormal embryos, and plantlets as described previously. Percent of embryos converting into plantlets was calculated as % plantlets = plantlets / (bipolar embryos + plantlets) x 100. The study was conducted as a separate experiment for each explant-derived calli source as a randomized complete blocks design (RCBD) 2 factor factorial with 10 auxin treatments (i.e. 5 levels each of 2,4-D and NAA) x 4 kinetin levels x 6 Petri dishes as replications. Such an experimental design was conducted in both IM and MM. Mean separation was performed using LSD (Steel and Torrie, 1980).

In order to determine whether the abnormal formation of embryos observed in MM was due to a carry-over of auxin from IM to MM, the following experiment was conducted: IVC-derived calli of male line A-9 were placed on IM containing (ppm): 2,4-D (1.5) + kinetin (0.1), 2,4-D (0.3) + kinetin (0.1), or NAA (1.5) + kinetin (0.1), NAA (0.3) + kinetin (0.1), for 4 weeks. Calli were subsequently transferred to MM and subcultured every 4 weeks during a 6 month period. Prior to each subculture, callus pieces were withdrawn randomly and weight and the number of abnormal and normal

embryos and plantlets were counted. Analysis of variance (AOV) and means were calculated from 6-8 replications (100 x 15mm Petri dishes) per treatment.

RESULTS

Spear section (SS)-derived callus

Callus was formed at the periphery of SS 4-8 weeks after placement on MS with 2,4-D or NAA and kinetin alone or in combination (0-10ppm). Segments from the upper part of the spear (4-12 internodes) formed callus more rapidly than those from the lower segments (14-22 internodes). Media of MS + 2,4-D (0.5-2ppm) and/or NAA (1-3ppm) + kinetin (0-1ppm) were optimal for callus initiation. On MS + 2,4-D (0.5-2ppm) and kinetin (0-1ppm) the callus was compact, yellowish and mucilaginous with a high frequency of globular translucent embryoids. These embryoids consisted of small cells with a dense cytoplasm surrounded by large vacuolated cells; whereas, on MS + NAA (1-3ppm) and kinetin (0-1ppm) callus was yellowish and friable with sporadic globular embryoids surrounded by large vacuolated cells. Explants of SS placed on MS + low 2,4-D or NAA levels (0-0.2ppm) or on MS + kinetin alone (0-1ppm) gave rise to sparse, non-vigorous callus.

The interaction of auxin x kinetin was highly significant for both % callus growth and the number of globular embryos on IM (Table 1). On IM with kinetin (0-1ppm), fresh weight of callus did not increase as 2,4-D levels increased from 0 to 0.1ppm. The fresh weight of

callus gradually increased at 0.1 to 1ppm and decreased at 10ppm. On IM with the same kinetin levels but with NAA, callus fresh weight did not increase much as NAA increased from 0 to 0.1ppm, sharply increased between 0.1 to 1ppm and decreased at 10ppm (Table 2, Fig.1A). Calli slightly increased as 2,4-D or NAA levels increased from 0 to 0.1 ppm; sharply increased at 0.1 to 1 ppm and decreased at 10 ppm (Table 2, Fig. 1A). The combinations of 2,4-D or NAA (1ppm) with kinetin (0-1ppm) gave the highest callus increase (341-395%) (Table 2, Fig. 1A). A high kinetin level (10 ppm) slightly increased callus growth mainly when media contained low NAA levels (0-0.1ppm) but in combination with 2,4-D (0.1-1 ppm) it reduced growth (Table 2, Fig. 1A). Kinetin alone (10ppm) mainly affected morphogenesis, where green hard callus with some shoot formation occurred.

The absence of auxin or low levels of auxin (0, 0.01, and 0.1 ppm) in combination with all kinetin levels only induced a low frequency of small globular embryos (Table 2, Fig 1B). High 2,4-D levels (1 and 10 ppm) in combination with kinetin (0-1 ppm) induced a 30-50 fold higher frequency; whereas, the highest kinetin (10 ppm) and 2,4-D (10ppm) combination inhibited their formation (Table 2, Fig. 1B). High NAA levels (1-10ppm) also increased the frequency of globular embryos, but the effect was not as pronounced as that of 2,4-D; (Table 2, Fig. 1B). SS-derived callus had only a few large embryoids on IM (Table 2, Fig. 1B).

The interaction of auxin x kinetin in IM was significant in affecting the number of bipolar embryos formed by SS-derived calli on MM (Table 1). Low levels of 2,4-D or NAA (0-0.1 ppm) with kinetin (0-10 ppm) in IM gave rise to only a few (2-11/g callus) bipolar embryos on MM (Table 2, Fig. 1C), while higher auxin levels (1 and 10ppm) significantly increased the frequency of such embryos; by 5-40 fold (2,4-D) and 2-5 fold (NAA) (Table 2, Fig. 1C). However, 2,4-D also gave rise to a high number of abnormal embryo structures that resembled fused elongated roots, and only a few typical embryoids that gave rise to plantlets (Fig. 1C and D). In this experiment, SS-derived callus gave rise to a very low frequency of embryos with the capacity to convert into plantlets following treatment with the various IM (Fig. 1D). However, in a separate experiment (with male line A-9), SS-derived callus gave rise to a higher conversion rate (11%) following transfer from MS + (ppm): 2,4-D (0.5-3) and kinetin (0.5) to MS + (ppm): NAA (1.0) and kinetin (0.3) (Appendix).

Lateral bud (LB)-derived callus

Within four weeks on the initiation medium of MS + (ppm): NAA (0.1) and kinetin (0.01), LB gave rise to a hard compact callus that simultaneously formed roots with a yellow friable callus and globular embryos. The friable callus portion was subcultured twice at four weeks intervals; it proliferated vigorously, forming new embryos while mature embryos developed into plantlets. At the end

of the second subculture stage, friable portions of the callus were transferred to IM.

Auxin and kinetin concentrations alone had a significant effect on callus growth on IM, but the interaction was not significant (Table 1). The combination of 2,4-D or NAA at 1ppm with kinetin (0-1 and 0-10 ppm, respectively) gave the highest callus increases (431-471% and 439-514%, respectively) (Table 3, Fig. 2A). There was no difference between kinetin levels (0-1ppm) in affecting callus growth on IM with low auxin levels (0-0.1ppm) (Table 3, Fig. 2A). However, in combination with high auxin levels (1 and 10ppm), high kinetin level (10ppm) reduced callus growth (Table 3, Fig. 2A).

The interaction of auxin x kinetin was highly significant in affecting the frequency of globular and bipolar embryos on IM (Table 1). There was no difference in the frequency of globular embryos on IM with low auxin levels (0-0.1 ppm) in combination with kinetin (0-1ppm); 28-50 embryos/g callus, while high kinetin (10 ppm) reduced their frequency; 15-19/g callus (Table 3, Fig. 2B). High auxin levels (1 and 10 ppm) in combination with kinetin (0-1ppm), sharply increased the frequency of globular embryos; by 3.1-10 fold(NAA), and 5-15 fold(2,4-D), whereas, high kinetin (10ppm), particularly in combination with high auxin (1 and 10ppm) inhibited their formation (Table 3, Fig. 2B). High kinetin (10ppm) had mainly a morphogenetic effect as it negated embryogenesis (Figs. 2B, C and D) while inducing

greening of calli and some shoot formation. Calli derived from LB cultured on IM with auxin (0-0.1ppm) and kinetin (0-1ppm) formed 17-21 bipolar embryos/g callus. High auxin (1 and 10ppm) and kinetin levels (10ppm) reduced the number of bipolar embryos but not significantly (5-13 embryos/g callus; Table 3, Fig. 2B). High auxin levels inhibited embryo growth, leading to a reduction in number of bipolar embryos while enhancing the formation of new globular embryos (Table 3, Fig. 2B).

The interaction of auxin x kinetin levels in IM was highly significant in affecting the frequency of bipolar embryos and their conversion rate into plantlets on MM (Table 1). Low auxin levels (0-0.1ppm) in combination with kinetin (0-1ppm) in IM induced only a low frequency of bipolar embryos on MM (between 17-27 per gram callus), while high levels of 2,4-D and NAA (1 and 10ppm) in combination with low kinetin levels (0-0.1 and 0-1ppm, respectively) sharply increased their frequency by 1.3-3.5 fold (Table 3, Fig. 2C). High kinetin (10ppm) sharply reduced the number of embryos and their conversion into plantlets on MM (Table 3, Fig. 2D). Induction with various 2,4-D levels (0-10 ppm) or with low NAA levels (0-0.1) in combination with kinetin (0-1ppm) gave rise to 1.7-4.7 plantlets/g callus (12.5-22.3%) on MM, while induction with NAA at levels of 1 and 10ppm in combination with low kinetin levels (0-1 ppm), resulted in a higher number of plantlets; 8.3-10.7/g callus (14-21%) (Table 3, Fig. 2D). High 2,4-D levels (1 and

10ppm) induced the formation of abnormal embryos similar to those described for SS-derived callus. This resulted in a low conversion rate (1.3-3.7 plantlets/g callus; 2.3-5.7%) (Table 3, Fig. 2D).

In Vitro Crown (IVC)-derived callus

IVC-derived callus placed on MS + various levels of NAA and kinetin (0-1 ppm) occasionally formed a compact organized callus with shoot and root initiation, and simultaneously a yellowish friable embryogenic callus. The latter also consisted of embryos at various developmental stages together with a few plantlets. This callus was subcultured twice at four week intervals, and the fine friable portion of the calli was used for the experiment on IM.

There was no interaction between auxin and kinetin with respect to callus growth on IM (Table 1). On IM the fresh weight of callus slightly increased as auxin levels increased from 0 to 0.1 ppm, sharply increased at 0.1 to 1 ppm, and decreased at 10 ppm. The combination of 2,4-D or NAA (1 ppm) and kinetin (0-1 ppm) gave rise to the highest callus increases (508-623 and 582-620%, respectively). High kinetin (10 ppm) reduced callus growth. This was particularly evident in combination with 2,4-D or NAA at levels of 0-1ppm (Table 4, Fig. 3A).

The interaction auxin x kinetin was highly significant with respect to the frequency of globular and bipolar embryos on IM (Table 1). IVC-derived callus formed a

relatively large number of embryos in various developmental stages on IM with low auxin levels (0-0.1 ppm) and kinetin (0-1ppm) (18-37 globular and 59-94 bipolar embryos/g callus; Table 4, Fig. 3B). However, higher auxin levels (1 and 10ppm) sharply increased the number of globular embryos, while decreasing the number of bipolar embryos, and high kinetin (10 ppm) inhibited embryo formation while (Table 4, Fig. 3B) inducing greening and shoot formation.

The interaction of auxin x kinetin in IM was significant with respect to the number of bipolar embryos and mature plantlets formed on MM (Table 1). Callus transferred from IM with low auxin levels (0-0.1ppm), gave rise to embryos in various developmental stages; 48-84/g callus (Table 4, Fig 3C). Higher levels of 2,4-D (1 and 10 ppm) or NAA (10 ppm) in combination with kinetin (0-1 ppm) increased the frequency of such embryos on MM; by 1.3-2.8 fold (2,4-D) and 1.2 fold (NAA) (Table 4, Fig. 3C). Low auxin levels (0-0.1ppm) in combination with kinetin (0-1 ppm) gave rise to 7.2-11.7 plantlets/g callus (9-17.7%) on MM. High 2,4-D (10ppm) reduced it to 1.7-2.7 plantlets/g callus (1.1-2.1%), while NAA (1 and 10ppm) slightly increased it to 9.8-13.7 plantlets/g callus (9-17.3%) (Table 4, Fig. 3D). Induction with high kinetin (10ppm), significantly reduced embryo formation and conversion rate to plantlets on MM (Table 4, Figs. 3C and D).

Continuous subculture of embryos on MM at two-three weeks intervals, during a four to six month period, enhanced

the formation of new embryos and their conversion into plantlets. This was mainly in LB and IVC-derived callus induced with NAA (10ppm); 4-13 plantlets/g callus in each subculture.

On MM mostly typical embryos were formed. However, abnormal embryos in various frequencies were also observed (Fig. 4). Such embryos may have developed a cotyledon and radical, but had an undeveloped shoot meristem. On the other hand, some embryos had more than one shoot meristem (3-4) developing at the same time. In addition, fully developed embryos were partly fused or clustered with each other. Continual subculture of callus of line A-9 every 4 weeks on MM reduced the frequency of abnormal embryos while it slightly increased the number of embryos that converted into plantlets (Fig. 4).

Table 1. AOV for callus increase and frequency of somatic embryos and mature plantlets on IM and MM for three asparagus explants.

SS-derived callus

Source	DF	IM			MM	
		Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
R	5	NS	NS	NS	NS	NS
A	9	**	**	*	**	*
K	3	*	**	**	**	NS
A x K	27	*	**	NS	**	NS

B-derived callus

Source	DF	IM			MM	
		Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
R	5	NS	NS	NS	NS	NS
A	9	**	*	*	**	**
K	3	*	**	**	**	**
A x K	27	NS	*	**	**	**

IVC-derived callus

Source	DF	IM			MM	
		Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
R	5	NS	NS	NS	NS	NS
A	9	**	**	**	**	**
K	3	**	**	**	**	**
A x K	27	NS	**	**	*	*

**, *, NS, significant at the 1%, 5% levels, or not significant, respectively.

R = Replications
A = Auxin in IM
K = Kinetin in IM

Table 2. Responses of SS-derived callus on IM and MM. The effect of 2,4-D or NAA and kinetin on callus growth (%) and number of globular and bipolar embryos after 4 weeks on IM; and bipolar embryos and plantlets after 6 weeks on MM.

		IM			MM	
2,4-D (ppm)	Kinetin (ppm)	Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
0	0	109	5	8	7	0.3
	0.1	86	6	3	7	0.3
	1	190	8	9	9	0.5
	10	178	8	11	7	0.5
0.01	0	136	5	7	3	0
	0.1	133	7	4	8	0.5
	1	206	6	10	8	0.3
	10	146	7	8	8	0.2
0.1	0	295	13	6	2	0.2
	0.1	243	8	6	5	0.3
	1	244	14	8	8	0.2
	10	109	15	8	9	0.3
1	0	392	284	4	82	1.8
	0.1	341	312	2	77	1.5
	1	366	314	7	50	0.8
	10	276	49	6	29	0.8
10	0	264	491	3	147	1.2
	0.1	261	479	2	180	2.0
	1	269	489	4	161	1.3
	10	221	180	8	67	1.8
NAA (PPM)						
0	0	99	2	8	10	0.5
	0.1	81	3	8	10	0.2
	1	164	6	6	7	0.3
	10	207	6	3	10	0.5
0.01	0	88	5	8	8	0.2
	0.1	118	3	4	9	0.2
	1	125	8	9	9	0.3
	10	185	7	4	11	0.2
0.1	0	95	4	8	7	0.3
	0.1	129	3	5	10	0.3
	1	142	10	9	5	0.2
	10	228	6	2	8	0.2
1	0	344	33	8	17	0.7
	0.1	370	36	3	23	0.7
	1	395	47	8	24	0.8
	10	385	23	4	14	0.3
10	0	256	95	3	27	1.5
	0.1	239	84	2	35	1.3
	1	239	88	6	37	1.3
	10	294	65	2	22	0.8
L S D	(1%)	101	41	6.4	26.5	1.4

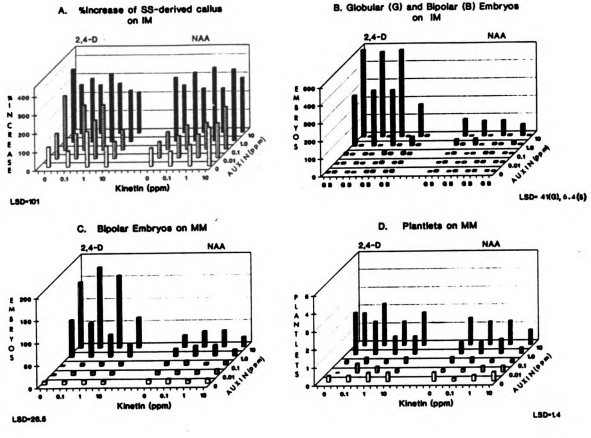

Fig. 1. Responses of SS-derived callus on IM and MM: A) % callus growth and B) Number of globular embryos (<3mm) and bipolar embryos (4-7mm) after 4 weeks on IM, C) Number of bipolar embryos and D) plantlets after 6 weeks on MM.

Table 3. Responses of LB-derived callus on IM and MM. The effect of 2,4-D or NAA and KIN on: % callus growth, number of globular and bipolar embryos after 4 weeks on IM; and bipolar embryos and plantlets after 6 weeks on MM.

		IM			MM	
2,4-D (ppm)	Kinetin (ppm)	Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
0	0	206	39	21	22	3.6
	0.1	208	48	19	20	3.6
	1	217	30	17	23	3.3
	10	201	16	10	4	0.3
0.01	0	191	48	21	20	4.1
	0.1	186	50	19	22	4.5
	1	217	28	18	23	3.0
	10	189	19	10	4	0.3
0.1	0	286	47	20	24	4.0
	0.1	242	49	20	23	4.2
	1	321	31	17	23	3.7
	10	220	18	10	4	0.3
1	0	431	265	13	68	3.7
	0.1	414	241	13	68	1.8
	1	471	230	10	32	1.7
	10	352	29	7	6	0.3
10	0	325	433	6	52	2.3
	0.1	277	448	7	41	1.3
	1	242	450	5	48	2.0
	10	249	198	8	23	1.2
NAA (PPM)						
0	0	242	41	20	28	3.5
	0.1	276	38	19	25	4.5
	1	185	39	19	17	3.3
	10	191	17	8	6	0.5
0.01	0	229	48	20	24	3.5
	0.1	251	45	18	25	4.5
	1	212	35	20	19	3.2
	10	225	17	9	6	0.7
0.1	0	217	47	19	27	4.2
	0.1	216	48	18	26	4.7
	1	220	31	16	21	3.7
	10	266	15	9	7	0.7
1	0	504	133	20	41	9.2
	0.1	574	168	13	45	8.3
	1	472	170	10	51	10.5
	10	439	37	7	4	0.5
10	0	343	294	13	58	8.5
	0.1	310	280	9	60	9.2
	1	266	306	7	71	10.7
	10	217	75	5	27	3.5
LSD	(1%)	112	49	15.4	9.3	2.3

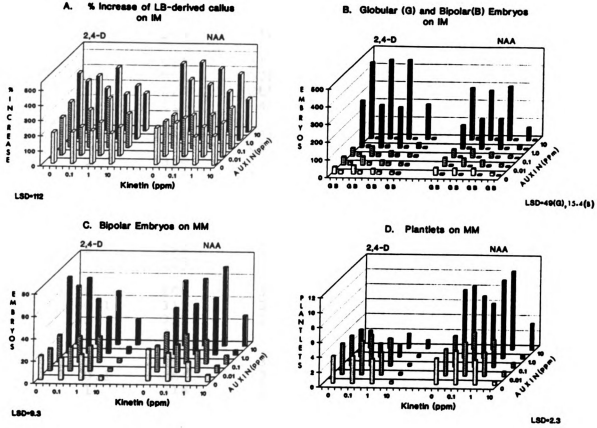

Fig. 2. Responses of LB-derived callus on IM and MM: A) % callus growth and B) number of globular and bipolar embryos after 4 weeks on IM, C) Number of bipolar embryos and D) plantlets after 6 weeks on MM.

Table 4. Responses of IVC-derived callus on IM and MM as % callus growth, formation of globular and bipolar embryos after 4 weeks on IM. Number of bipolar embryos and plantlets after 6 weeks on MM.

		IM			MM	
2,4-D (ppm)	Kinetin (ppm)	Callus (%)	Globular (<3mm)	Bipolar (4-7mm)	Bipolar (4-7mm)	Plantlets
0	0	259	30	73	63	9.3
	0.1	207	30	83	71	7.7
	1	306	18	63	48	7.2
	10	195	9	22	15	1.7
0.01	0	289	31	83	70	11.3
	0.1	211	31	91	67	7.7
	1	323	26	65	53	8.2
	10	180	10	16	12	0.7
0.1	0	312	35	87	75	10.1
	0.1	297	33	94	84	10.2
	1	353	26	66	70	8.7
	10	209	10	17	13	0.7
1	0	508	317	15	118	7.2
	0.1	531	372	15	119	7.7
	1	623	288	19	86	5.8
	10	414	49	12	42	1.7
10	0	319	449	8	165	1.7
	0.1	374	467	6	142	2.0
	1	356	400	6	143	2.7
	10	294	158	4	108	2.7
NAA (PPM)						
0	0	276	28	89	71	9.0
	0.1	314	36	83	75	9.2
	1	294	30	74	73	7.3
	10	223	8	23	21	1.3
0.01	0	266	32	91	69	10.0
	0.1	319	33	89	84	9.2
	1	322	21	59	68	7.8
	10	232	7	16	18	4.7
0.1	0	297	37	78	79	9.2
	0.1	364	34	90	84	11.7
	1	318	20	74	75	8.0
	10	247	7	16	15	1.2
1	0	582	114	44	78	13.0
	0.1	601	133	37	86	13.3
	1	620	110	37	81	11.8
	10	447	23	8	26	1.5
10	0	367	219	22	99	10.0
	0.1	390	251	21	96	9.8
	1	406	226	20	107	13.7
	10	334	117	8	52	1.5
LSD	(1%)	133	50	17.9	25.4	5.2

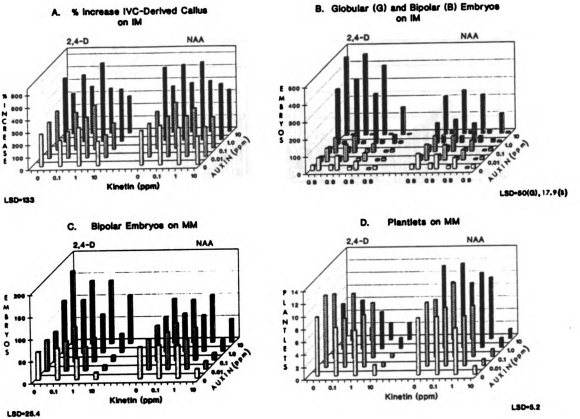

Fig. 3. IVC-derived callus on IM and MM: A) % callus growth and B) number of globular and bipolar embryos after 4 weeks on IM, C) Number of bipolar embryos and D) plantlets after 6 weeks on MM.

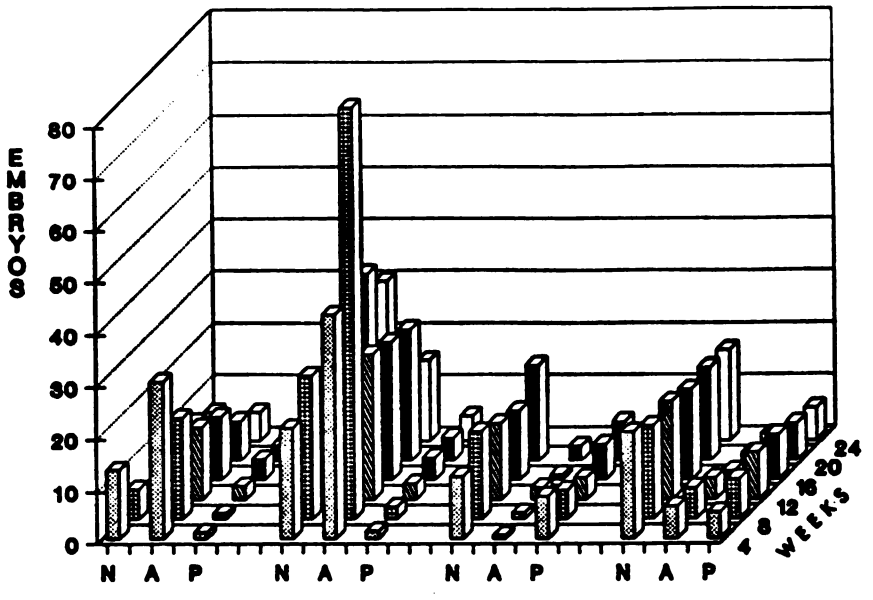

Fig. 4. Number of normal (N) and abnormal (A) embryos (4-7mm) and plantlets (P) formed during 6 months on MM, counting and subculture every 4 weeks, following induction on MS + (ppm) (left to right): 1) 2,4-D (0.3) and kinetin (0.1), 2) 2,4-D (1.5) and kinetin (0.1), 3) NAA (0.3) and kinetin (0.1), 4) NAA (1.5) and kinetin (0.1).

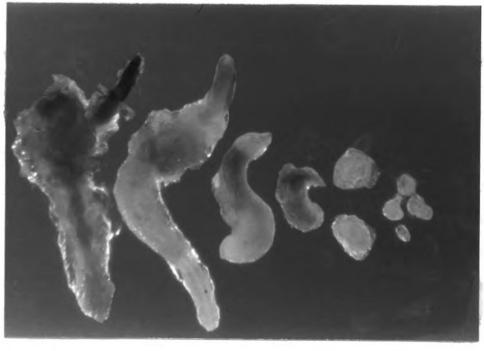

Fig. 5. Somatic embryos at different developmental stages formed in IVC-derived callus on MM (six weeks), following induction with MS +(ppm) NAA (10) and kinetin (0.1). (x 14).

DISCUSSION

The main goal in this research was to establish an efficient protocol for somatic embryogenesis of tissues derived from mature asparagus crowns by examining the role of explants and phytohormones. Indeed, explant source and hormonal conditions in the initial culture medium were found to significantly influence callus initiation and subsequent rate of embryogenesis. The SS were relatively slow to form callus; whereas, LB and IVC readily formed embryogenic calli on MS media with low auxin levels. This observation is similar to that seen in other studies, which showed that explant type and its developmental stage are important for the establishment of embryogenic callus (Bapat and Rao, 1979; Gharayal and Maheshwari, 1981; Vasil, 1987; Wenzler and Meins, 1986; Wilson and Street, 1975). Explants with meristematic cells usually have a higher competence to form embryogenic callus than those taken from mature organs with highly differentiated cells (Vasil, 1987). It is likely that in asparagus the LB and IVC explants have a higher frequency of such meristematic cells than SS which consisted mainly of highly differentiated vascular tissue. Additionally or alternatively, LB and IVC may have higher levels of endogenous hormones which activate the proliferation of embryogenic callus.

Rajasekaran et al. (1987) reported that embryogenically competent tissues in young leaves of Pennisetum purpureum, and their derived embryogenic callus, contained high

endogenous levels of indoleacetic acid (IAA) and abscisic acid (ABA), whereas, mature parts of leaves, which do not form embryogenic calli, and non-embryogenic calli contained 5-20 and 3-4 fold lower levels of these phytohormones, respectively. LB- and IVC-derived calli placed on IM devoid of/or with low 2,4-D or NAA levels formed embryos of various developmental stages and plantlets. However, when such calli were placed on IM with high auxin levels (1 and 10 ppm) embryo development beyond the globular stage was arrested. Simultaneously, the formation of new, small globular embryoids was enhanced. Induction with auxin was found to foster differentiation of cells and development of cytoplasmic globular proembryos (Ammirato, 1986; Halperin, 1966; McWilliam et al. 1974; Street and Withers, 1974). Sung (1979) reported that high auxin levels suppressed embryogenesis in carrot, and Kochba and Spiegel-Roy (1977) reported that endogenous IAA inhibited somatic embryogenesis in habituated callus of shamuti orange, (Citrus sinesis) while inactivation of IAA enhanced embryogenesis. Kochba et al. (1977) concluded that low endogenous auxin levels are favorable to maintain a low frequency of embryogenesis in the habituated callus. Changes in the levels of endogenous auxin are also linked to somatic embryogenesis in carrot cells (Fujimura and Komamine, 1979).

. The formation of embryogenic callus from IVC and LB and its proliferation on media devoid of auxin or with low auxin levels while giving rise to bipolar embryos and plantlets,

implies that these explants and their derived calli may have levels of endogenous auxin that are sufficient to induce embryo formation while low enough to enable the continual development of embryos. With exogenous auxin (1 and 10 ppm) the developmental pattern was shifted more towards synchronous formation of globular embryos while inhibiting further embryo development. The SS-derived callus may lack such endogenous phytohormones and/or cells with embryogenic capacity. Thus, higher levels of exogenous auxin were necessary to induce embryogenesis from SS-derived callus. Giberelic acids that may activate the formation of excessive callus were detected in asparagus shoots established in vitro (Khunachak et al. 1987), and auxin and cytokinins in various levels were detected in different parts of asparagus plants (Rossi et al. 1989). The role of endogenous phytohormones in asparagus explants relative to efficacy of somatic embryogenesis still awaits evaluation.

The auxin 2,4-D (1 and 10 ppm) induced a high frequency of embryos, but it also increased the formation of abnormal structures. Conversely, NAA of the same levels induced a lower embryo frequency but it increased the rate of embryos that converted into plantlets; mainly in IVC and LB-derived callus. Continual MM subculture of callus induced with 2,4-D, led to a decrease in the formation of abnormal structures and to a slight increase in conversion of embryos into plantlets. This indicated that the abnormal structures may have resulted from a carry-over effect of 2,4-D to MM. NAA

induced fewer abnormalities than 2,4-D; it is therefore probable that the carry-over effect of NAA is minimal or, at least, less than that of 2,4-D. However, embryogenic callus that has been induced with low levels of auxin also formed some abnormal structures.

This points to the possibility that abnormal structures result not only from carry-over of auxin to MM, but also from other genetic, epigenetic and physiological factors which lead to alteration or to incomplete development of the embryos (Ammirato, 1986; Raghavan 1986; Vasil, 1987). Abnormal formation of embryos has been reported in various species capable of somatic embryogenesis (Ammirato, 1986).

In most species, and especially in monocotyledons, 2,4-D is considered the most potent auxin for the induction of callus and formation of somatic embryos (Ammirato, 1986; Vasil et al. 1982). The conversion of embryos to plantlets due to transfer of SS-derived callus from induction medium with 2,4-D to medium with NAA followed by transfer to MM with a lower NAA level, demonstrates that in asparagus 2,4-D may be important in initiating embryonic callus from SS. Apparently, NAA has a higher capacity to induce normal embryogenesis at a later stage of culture. Stewart and Mapes (1971) used a series of sequential treatments, where stem segments of mature asparagus plants were induced to form callus in Whites medium supplemented with CM and NAA. The NAA was replaced by 2,4-D for induction of callus in suspension. Subsequently, this medium was substituted with a

high salt concentration and NAA for induction of embryoids, followed by media supplemented with CM and IAA for embryoid development. On the other hand, Wilmar and Hellendoorn (1968) reported that 2,4-D (1ppm) and kinetin (0.3ppm) were sufficient to induce embryogenic callus from the hypocotyl of asparagus seedlings. Reuther (1977) demonstrated somatic embryogenesis in asparagus by placing shoot tip and shoot segments on LS + NAA (1.0ppm) and KIN (1.0ppm) to form callus. This was followed by induction on LS + IAA (1.0ppm) and BAP (0.1ppm) for organogenic callus and embryoid formation; replaced by LS or LS + (mg/L): IAA (0.5) for embryoid development. The present study indicates that it was sufficient to use NAA for induction of somatic embryogenesis in explants from mature asparagus. However, it should be taken into account that various genotypes may have different requirements for auxin type and concentration (Brown, 1988).

Induction with kinetin alone (0-1 ppm) did not have a significant effect on asparagus callus growth, induction with high kinetin levels negated embryogenesis. Such an inhibitory effect has also been reported in other monocotyledonous species (Dale and Dembarogio, 1979). In a separate experiment (limited data) we observed that MM with kinetin or 2iP (0.3-0.8 ppm) and NAA (0.1 ppm) increased the frequency of plantlets (from 14% to 17.5%) arising from embryogenic callus derived from IVC, as compared with MM with NAA (0.1ppm) alone. It has been suggested that changes

in cytokinin levels against a constant level of auxin may play a role in embryo development and maturation (Williams and Collin, 1976; Al-Abta and Collin, 1979).

This study demonstrates that clonal propagation through somatic embryogenesis in asparagus is feasible when different explants and growth regulators are used. The IVC and LB were found to be better sources than SS for embryogenic callus, and NAA was superior to 2,4-D (1-10ppm) in induction of normal embryos. The IVC cultured on MS + (ppm): NAA (0.1) and kinetin (0.01) sporadically formed embryogenic callus. Induction of such callus mainly on MS + NAA (10ppm) enhanced embryogensis and formation of normal mature plantlets on MM.

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SECTION II

ANATOMY AND MORPHOLOGY OF *ASPARAGUS OFFICINALIS* L. SOMATIC EMBRYOS

ABSTRACT

The developmental histology and morphology of asparagus somatic embryos arising in callus was examined and contrasted with that documented for zygotic embryos. Histological sections of lateral bud (LB)-derived callus cultured for two weeks on induction medium (IM) of Murashige and Skoog salts and vitamins (MS) + NAA (1.5ppm) and kinetin (0.1ppm), showed the formation of cytoplasmically dense, small embryogenic cells arranged in distinct groups of 2-12 cells. After four weeks on IM, the formation of small globular embryos, 8-32 cells, with large suspensor cells (8-14) were observed. In addition, large globular embryos, 0.8-3mm diam, in a late developmental stage and a few embryos in the early bipolar stage were found at the surface of the callus. Histological sections taken from spear segment (SS)-derived callus on IM of MS + (ppm): 2,4-D (0.5-1.5) and kinetin (0.1), revealed embryonic cells and globular embryos in a higher frequency (1.5-2 fold) than in LB-derived callus. In addition, many spherical unorganized meristematic domes with numerous small cytoplasmically dense cells occurred in SS-derived calli. Upon transfer of such calli to maturation medium (MM) of MS + NAA (0.05ppm) and kinetin (0.1ppm), the meristematic domes gave rise to abnormal embryogenic structures. Within two weeks culture

of SS and LB-derived calli on MM, globular embryos developed a bipolar shape having an expanded upper portion which formed the cotyledon and a smaller portion which formed the hypocotyl and radical base. Mature bipolar embryos, 4-6 weeks on MM, had a distinct shoot apex at the junction of the cotyledon and the hypocotyl and vascular tissue connecting the radical, shoot apex and the cotyledonary portion. Most of the somatic mature embryos resembled the asparagus zygotic embryos having a crescent shape. Other somatic embryos had a round, wide cotyledon, typical of zygotic embryos of other monocotyledon species. Both embryo types gave rise to plantlets at equal rates.

INTRODUCTION

Somatic embryogensis has been reported in an increasing number of monocot species (Vasil 1982, 1983; Raghavan, 1986). Asparagus officinalis L. was one of the first such species to be regenerated to plantlets in vitro (Wilmar and Hellendoorn, 1968; Steward and Mapes, 1971; Reuther, 1977). Recently, Levi and Sink (1989) examined the role of explant sources and growth regulators in promoting asparagus somatic embryogensis. Calli derived from lateral buds (LB) and in-vitro established crowns (IVC) showed a higher embryogenic capacity than callus derived from spear sections (SS). Induction of these calli with 2,4-D or NAA gave rise to small globular embryos (<3mm diam) in a high and low frequency, respectively. However, embryogenic calli induced

with 2,4-D gave rise to many abnormal structures and to only a few normal bipolar embryos. Conversely NAA gave rise to mainly normal somatic embryos and plantlets on maturation medium (MM). This paper presents a histological study that was undertaken to define the developmental events that resulted in abnormal and normal embryo morphologies.

Similar studies of somatic embryogensis have been carried out in many different monocot species (Guiderdoni and Demarly, 1988). In asparagus, the gross morphology of the crescent-shaped somatic embryo stage (Bui Dang Ha et al. 1975) and a schematic pattern of the multicellular origin of embryos (Reuther 1983) have been reported. However, the developmental sequence of such embryos has not been histologically detailed as it has for the asparagus zygotic embryo (Riviere, 1973). As a result, shoot or root meristems found in asparagus callus cultures may be misinterpreted as embryos due to their similar shape and size (Reuther, 1983). In order to elucidate the asparagus somatic embryo developmental pattern, the anatomy of such embryos was examined and contrasted with that already observed by Riviere (1973) for zygotic embryos.

MATERIALS AND METHODS

Spear X-sections (SS) (3-5mm) and lateral buds (LB) (1-2.5cm) were obtained from the upper portion of 4-7 day old spears of C-3, a male asparagus crown selection, taken from greenhouse plants. The spears were surface sterilized

for 30 min with aqueous sodium hypochlorite (1.5% w:v) containing 2 drops of Tween-20 per 100 ml. Subsequently, spears were thoroughly rinsed with 3 changes of sterile distilled water. To initiate callus, SS were placed on Murashige and Skoog (1962) salts and vitamins medium (MS) + 2,4-D (0.5ppm) and kinetin (0.1ppm), while LB were placed on MS + NAA (0.1ppm), and kinetin (0.01ppm); 3 explants in each of ten 15 x 100mm Petri dishes which contained 25ml medium. Within four weeks primary calli were separated from the explants and subcultured twice at four week intervals. SS-derived calli were transferred to induction medium (IM) of MS + 2,4-D (0.5-1.5ppm) and kinetin (0.1ppm), while LB-derived calli were transferred to IM of MS + NAA (1.5ppm) and kinetin (0.1ppm). Following 4 weeks on IM, calli derived from both explants were transferred to maturation medium (MM) of MS + NAA (0.05ppm) and kinetin (0.1ppm). All culture media were solidified with 0.9% Bacto agar containing 3% sucrose and were adjusted to pH 5.9 prior to autoclaving. Initial and IM cultures were incubated in the dark at 26C; during MM they were held under a 16h photoperiod (32 uEm^{-2} sec^{-1}) from cool white fluorescent bulbs. For histological studies, 12-18 tissue samples (1.5-2cm diam) were taken after two and four weeks on IM, and after one, two, four and six weeks culture on MM. The tissues were embedded in paraffin following fixation in Craf III solution (Sass, 1951), dehydration in a tertiary butanol series, and clearing in an ethanol-xylol series (Berlyn and

Miksche, 1976). Sections were cut at 15 uM using a rotary microtome and stained in safranin-fast green and mounted with DPX (Berlyn and Miksche, 1976).

RESULTS AND DISCUSSION

After two weeks on IM LB-derived callus was yellowish and friable and was comprised of many islands of small embryonic cells surrounded by large vacuolated cells (Figs. 1A and B). The embryonic cells had a dense cytoplasm, conspicuous nucleus, thickened cell wall, and irregular planes of division (Fig. 1A) which resulted in the formation of discrete groups of 2-12 cells. Such differentiation is typical of early somatic embryogenesis in other monocot species (Ho and Vasil, 1983). After four weeks on IM, embryogenic cells (Fig. 1B), early globular (Fig. 1C), late globular, and a few embryos in early bipolar stage (Fig. 1D) were observed on the callus surface.

Many of the embryogenic cell groups had a distinct segmentation due to one transverse division of one cell and one to three longitudinal division of the other (Fig. 1B). This pattern resembles the quadrant and the subsequent octet stage of the zygote of various monocot species, where the basal cell gives rise to a suspensor of about 6-10 cells prior to intense division of the 2-4 upper terminal cells to form the globular portion (Raghavan, 1976). Such early organization suggests that asparagus somatic embryos may arise from single embryogenic cells (Haccius 1978). Other

studies on somatic embryogenesis in monocots also pointed to such possible cell origins (Vasil and Vasil, 1982).

At an intermediate stage asparagus somatic embryos were comprised of an elongated suspensor of 8-14 large, cytoplasmically enriched cells and upper spherical body of 8-32 small cells (Fig 1C). The globular portion evolved into a large mass (0.8-3mm) of small cells each with a dense cytoplasm, large starch grains and a conspicuous nucleus.

Within 1-2 weeks after transfer of embryogenic callus to MM, the globular embryos had a bipolar shape (Fig. 1D). Embryos at this stage had an extended upper portion which formed the cotyledon and a lower part which formed the hypocotyl and radical (Fig. 1E). Mature embryos were opaque and attained a length of 4-7mm within 4-6 weeks on MM. They possessed a distinct cotyledon with large cells, a lateral shoot apex at the junction of the hypocotyl and the cotyledon, a coleoptile primordium at the upper region, and an elongated radical. As well, there was an organized vascular tissue with distinct xylem and phloem cells connecting the root, shoot meristem and cotyledon (Figs. 1F, H). However, many mature embryos had a well-defined shoot apex but a small cotyledon, while others had a large cotyledon, but lacked a well-defined shoot apex. Unlike the variable morphology of the cotyledon or the shoot apex, the root apex was invariably well organized and covered with a root cap (Fig. 1F).

Many mature somatic embryos (Figs. 2B and 2D) had a morphology that resembled zygotic embryos (Fig. 2C), having a banana-or crescent-shape with an elongated cotyledon and a hidden shoot apex (Riviere, 1973; Esau, 1976). In addition, many mature somatic embryos had a half heart-like shape, consisting of a short but wide cotyledon (Fig. 2A), which did not resemble the asparagus zygotic embryo, but is typical of other monocotyledon species (Raghavan, 1976). Bui Dang Ha et al. (1975) observed somatic embryos with a similar shape in regenerating protoplast-derived asparagus calli. Irrespective of shape, both somatic embryo types gave rise in equal frequencies to plantlets within 4-10 weeks on MM (Fig. 2E). When transferred singly to culture tubes, the plantlets produced an extensive root system and new fern growth.

SS-derived callus proliferating on IM (containing 2,4-D) was compact, yellowish, shiny, and consisted of small globular translucent embryonic bodies and small embryonic cells in higher frequency (1.5-2 fold) than in LB-derived callus (not shown). Histological sections of SS-derived callus taken after four weeks on IM revealed that in addition to the many embryogenic cells and globular embryos (at early and late developmental stages), a large region of callus formed many unorganized spherical meristematic domes (not shown). These were individual large masses composed of numerous small cells, each with a dense cytoplasm and conspicuous nucleus. The cells were smaller and denser at

the center of the globule which was covered with a few layers (5-8) of larger cells containing numerous small starch grains in the cytoplasm.

In contrast, globular embryos had larger cells (about 1.5-2.0 fold) that were distinctly separated from the surrounding large vacuolated cells. The meristematic domes gave rise to mostly abnormal embryo-like structures with numerous roots following culture on MM. Only a few normal-appearing bipolar embryos that formed on MM which subsequently gave rise to mature plantlets (3-5%). It is likely that the meristematic domes originated in a similar fashion as shown for globular proembryos (Figs. 1A-D). However, the presence of 2,4-D in IM appeared to have inhibited the embryogenic differentiation that occurred on IM with NAA (Fig. 1D).

It has been shown previously (Karelson and Vasil, 1986; Ammirato, 1986) that in auxin containing medium, proembryos enlarge and either bud-off or separate into additional proembryos. The excessive division and redifferentiation of embryonic cells in SS-derived callus on IM also led to abnormal embryo development on MM, i.e., fused embryos, fused and/or enlarged cotyledons. Morphological and developmental abnormalities during embryo maturation can often be traced to inconsistencies in the normal sequence of organizational events (Ammirato, 1986). It was confirmed here that such abnormal events occurred in the early developmental stages. These may have resulted from lack of

embryogenic capacity in SS-derived callus and/or the presence of 2,4-D. In a related study, It was found that NAA may be a more appropriate auxin than 2,4-D for the induction and maturation of asparagus somatic embryos (Levi and Sink 1989). Similarly, Gleddie et al. (1983) reported that NAA had a higher specificity in inducing somatic embryogensis in eggplant than 2,4-D or IAA.

The histological and morphological comparisons revealed homologies at the early developmental stages of asparagus somatic embryogenesis from callus and the pattern known for zygotic embryos *in vivo* of various monocot species (Raghavan, 1976), where an active suspensor with large and cytoplasmically enriched cells is formed prior to the development of spherical embryo. At later stages, most somatic embryos resembled asparagus zygotic embryos in having a crescent-shape (Riviere, 1973), while others had a short but expanded cotyledon typical of other monocots (Raghavan, 1976; Vasil et al., 1985).

The mature embryos gave rise to plantlets with a distinct cotyledon residue at the junction of the shoot and root (Fig. 2E). This resembled the asparagus seed embryo during germination where the lower region of the cotyledon elongates and pushes the radical and the apical meristem out from the seed, while the other end of the cotyledon remains embedded in the endosperm (Riviere, 1973; Esau, 1976). Additionally, the mature plantlets had an active lateral shoot meristem adjacent to the primary shoot (Fig. 2E),

similar to that of germinating zygotic embryo (Riviere, 1973).

Fig. 1 Developmental sequence of somatic embryos from LB-derived callus.

A. Callus on IM showing early formation of embryogenic initial cells. (x 310)

B. Suspensor formed in callus on IM. Note the longitudinal and transversal division of the apical and the basal cells. (x 310)

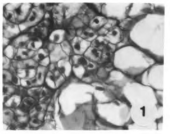
C. Early globular stage on IM. Note the large suspensor cells and globular embryo. (x 620)

D. Early bipolar shaped embryo on IM with differentiation of cotyledon and suspensor surrounded by layers of large vacuolated cells. (x 215)

E. A half heart-shaped embryo on MM with differentiated cotyledon, root meristem, and suspensor cells. (x 160)

F and H. Serial median longitudinal sections through an embryo in the late maturation stage; cotyledon, coleoptile, vascular system with xylem and phloem cells (x 16.5), and apical meristem (x 160) .

G. Longitudinal section through shoot apex of mature embryo; vascular system, coleoptile, first leaf and second leaf. (x 78)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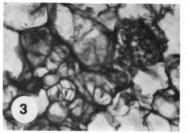
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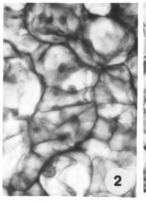
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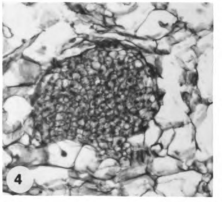
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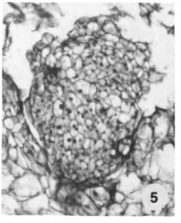
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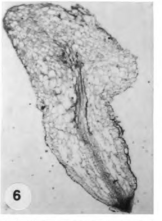
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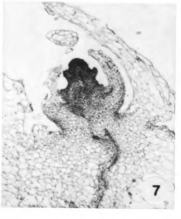
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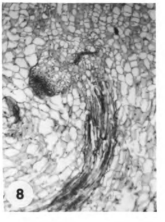
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Fig. 2.

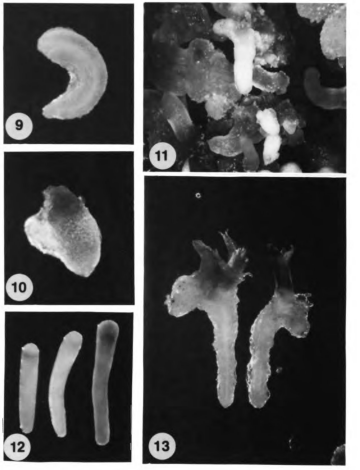
A. Mature embryo with well developed cotyledon, shoot primordia, and short radical; after 6 weeks on MM. (x 9.5).

B. Mature, elongated somatic embryo; after 4 weeks on MM. (x 14).

C. Zygotic embryo 4 weeks after anthesis. (x 16).

D. Embryonic LB-derived callus after 4 weeks on MM. (x 4.5).

E. Germinating somatic embryos showing cotyledon residue, and leaf primordia of lateral shoot meristem; after 6 weeks on MM. (x 9.5).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SECTION III

THE ROLE OF CARBOHYDRATE TYPE AND CONCENTRATIONS IN SOMATIC EMBRYOGENESIS IN *ASPARAGUS OFFICINALIS* L.

ABSTRACT

The role of carbohydrate (CHO) sources and concentration were studied during the induction and maturation stages of somatic embryogensis in asparagus. Glucose in induction media (IM); Murashige and Skoog salts and vitamins (MS) + 2,4-D (1.5ppm) significantly increased embryo formation in callus cultures, while sucrose had an intermediate effect and fructose was inhibitory, resulting in up to 1286, 719 and 515 globular embryos/g callus, respectively. However, in IM containing NAA (1.5ppm), the effect of glucose on embryo formation was not as pronounced, and there was no difference between glucose and sucrose, while fructose was inhibitory; up to 340, 310 and 183 globular embryos/g callus, respectively. Glucose (2%) in the subsequent maturation media (MM) of MS + (ppm): NAA (0.08) and 2iP (0.2) increased the formation of secondary small embryos, sucrose of the same level had an intermediate effect, while fructose reduced embryo formation; up to 438, 310 and 262 small globular embryos/g callus, respectively. With respect to morphogenesis, glucose (4-6%) promoted the formation of embryos with a large radical and retarded shoot formation, while fructose (2-5%) had the opposite effect, enhancing shoot formation while slowing root growth. Sucrose (3-6%) promoted the simultaneous elongation of shoot

and root apices. Fructose in MM enhanced the conversion of mature embryos into plantlets, while the combination of 5% sucrose in IM (containing NAA) and 5% fructose in MM had the highest conversion rate (10.2 plantlets/g callus) at the end of six weeks on MM. CHO concentration significantly effected the conversion rate of embryos into plantlets, where 2% gave the lowest while 5% had the highest rate; an average of 1.8 and 7.4 plantlets/g callus, respectively, following induction with NAA (1.5ppm). CHO type and concentration have an important role in normalizing somatic embryogensis in asparagus.

INTRODUCTION

As a carbon and energy source carbohydrates (CHO) are major constituents of plant tissue culture media, and sucrose is considered overall the best CHO for supporting growth and differentiation. However, few studies have examined the role of CHO types as alternatives for improving embryogenesis (Kononwicz and Janick, 1984; Spiegel-Roy and Saad, 1986; Strickland et al. 1987). Verma and Dougall (1979) reported that various CHOs matched, but were not superior to sucrose in their capacity to support embryogenic growth in carrot, and that embryo maturation on sucrose was slower than with the other sugars. Such a phenomenon was also observed in other systems where increased sucrose levels prevented precocious germination (Ammirato, 1983). Extensive studies with Theobroma cacao (Kononwicz and Janick, 1984) showed that low sucrose levels,

glucose or fructose all stimulated faster growth of somatic embryos than that of the zygotic embryo control. In embryonic cultures of citrus, CHOs such as malt extract, lactose, galactose and mainly glycerol improved the frequency of embryogenesis when compared to sucrose (Kochba et al, 1982; Ben-Hayyim and Neumann, 1983). Screening of alfalfa cultures for a compatible CHO in the presence or absence of sucrose (Strickland et al., 1987), showed that maltose, maltotriose and soluble starch improved the morphology and eventual conversion of somatic embryos to plantlets. CHOs were found to affect the morphogenesis of in vitro cultures per se or through their osmotic properties (Kishor, 1987; Lazzeri et al. 1988).

Indirect somatic embryogenesis from callus in vitro occurs in two distinct stages: induction (initiation) and embryo maturation (development). It is possible that the requirements for CHO type and concentration may differ depending on the embryogenic stages (Kononwicz and Janick, 1984; Thompson and Thorpe, 1987). In view of the relatively limited number of investigations of CHO requirements during somatic embryogenesis, and an interest in improving this system in Asparagus officinalis L., we examined the role of sucrose and its hexoses components glucose and fructose, at different levels in both induction and maturation stages. Sugar influence was monitored for embryo number, size and shape and rate of embryo conversion into normal plantlets.

It was demonstrated here that CHO type and level have a predominant role in normalizing somatic embryogenesis.

MATERIALS AND METHODS

Buds taken from 3-5 day-old spears of Michigan State University male crown A-9 were used as explants for the initiation of callus. Spears (10-18cm) were surface sterilized in dilut (1:3 v/v) aqueous Clorox (5.25% sodium hypochlorite) for 30 min, and rinsed (3x) with sterile distilled water. Excised lateral buds were placed (3-5 per Petri dish; 30 ml) on Murashige and Skoog (1962) basal medium (MS) containing 3% sucrose + NAA (0.5ppm) and 2iP (0.2ppm) and solidified with 0.9% (w/v) agar.

Within 4-6 weeks calli formed at the periphery of the explants. The calli were separated from the buds, and maintained over a 3 month period by subculture every 4 weeks. Subsequently, calli (0.4-0.6 g) were transferred to induction media (IM) of MS solidified with 0.9% (w/v) agar, and either supplemented with 2,4-D (1.5ppm) or NAA (1.5ppm) with 2iP (0.3ppm). Both IM were modified for individual CHO (glucose, fructose or sucrose) and concentrations, i.e. 2, 3, 4, 5, and 6% (w/v). After 4 weeks on IM, data on callus weight, embryo number, size and shape were collected; small spherical embryos (<3mm diam) were classified as globular embryos while large elongated (4-7mm length) as bipolar embryos. Callus growth was calculated: Callus increase (%) = (final weight-initial weight) x 100 /initial weight. Manual separation of calli using forceps afforded the counting of

embryos under a dissecting microscope. Next, calli on IM were selected at random and transferred to various maturation media (MM) of MS solidified with 0.9% Difco agar, and supplemented with NAA (0.08ppm) and 2iP (0.2ppm), and modified for CHOs as described previously. Calli (0.4-0.6g) were transferred to MM in 9 different sequence/combinations (Table 1). The same sugar level was maintained in both IM and MM of all sequence/combinations (i.e. 2%--->2%, 3%--->3% etc.). Thus, the experiment was conducted a randomized complete blocks design (RCBD) with 3 CHO types in IM x 3 CHO types in MM x 5 concentrations x 6 replications (replication = 3 callus samples per petri dish of 100 x 15mm).

Two separate RCBD experiments were conducted; one each for calli induced on IM containing 2,4-D or NAA. After 6 weeks on MM, data were collected on calli weight, number, size and shape of embryos, and number of mature plantlets. Conversion rate was calculated as follow: % plantlets= plantlets / (bipolar embryos + plantlets present in each random callus sample) x 100. Mean separations were determined using LSD (Steel and Torrie, 1980). While on IM, cultures were incubated at 27C in the dark, while on MM they were maintained at the same temperature with a photoperiod of 16h of light (16 uEm^{-2} Sec^{-1}) from cool white fluorescent bulbs.

RESULTS

Carbohydrate influence on growth and number of embryos on IM with 2,4-D

During its growth, the fresh weight of callus increased more when glucose levels were raised from 2 to 3% (495 to 630^) and decreased at 4 to 6% (622 to 359%). Callus fresh weight gain on IM with sucrose also increased 2 to 3% (402 to 501%) and decreased at 4 to 6% (458 to 378%). There was no significant difference between glucose and sucrose at these levels. With respect to fructose, callus fresh weight gain was highest at 2% (337%), and gradually decreased at 3 to 6% (315 to 227%; Fig. 1A).

Glucose induced the formation of granular callus containing numerous small (<1mm diam) dense globular embryo (Fig. 1B). Their number increased as glucose increased from 2 to 4% (809 to 1286 embryos/g callus), and decreased at 5 and 6% (1065 and 718/g callus), respectively. Sucrose induced a lower embryo formation than glucose. Embryo number increased as sucrose level increased from 2 to 5% (544 to 719/g callus), and decreased at 6% (559/g callus). Fructose induced the lowest number of embryos (Fig. 1B), but their number gradually increased as fructose increased from 2 to 6% (218 to 515/g callus; Fig. 1B). As compared to embryos on IM with glucose or sucrose, embryos on IM containing fructose were large, swollen and vitrified.

These results are similar to those in the preliminary study where a wider range of carbohydrate concentration (0-

9%) was used (appendix). However, there was little growth of callus (19-36%) and little embryo formation (0.5-2/g callus) on IM devoid of carbohydrates, and a reduction in callus growth occurres at the levels of 4 to 9%. Additionally, the number of small globular embryos increased with glucose and sucrose concentrations of 0 to 5% (appendix) but they were not as abandant as in the experiment described here.

Carbohydrate influence on callus growth and number of embryos on MM for calli derived from IM with 2,4-D

The interaction CHO type in IM x CHO type in MM x CHO concentration (I x M x C) was highly significant with respect to callus growth and number of embryos on MM (Table 1). Callus growth increased at higher CHO levels (Table 2, Fig. 2A). Calli transferred from IM containing fructose to MM with sucrose at a level of 3%, or to MM with glucose, fructose or sucrose at the level of 4%, had a higher growth than calli transferred from IM with glucose or sucrose of the same levels (Table 2, Fig 2A). For most calli, growth slowed or decreased as CHO levels increased from 5 to 6%. However, calli transferred from IM with glucose or fructose to MM with glucose, showed a continual increase at 6% (2916% and 2779%, respectively; Table 2, Fig. 2A).

The number of globular embryos was higher at lower CHO levels. It was highest (438/g callus) for calli transferred from IM with sucrose to MM with glucose at 2%, and gradually decreased as CHO concentration increased from 2 to 5% (Table

2, Fig. 2B). On the other hand, calli transferred from IM with glucose to MM with fructose at 2% had the lowest number of embryos (151/g callus). For this combination of CHOs, the number of globular embryos was similar at 2 to 4%, but decreased as CHO increased from 4 to 6% (Table 2, Fig. 2B).

Most calli showed a decrease in the number of globular embryos with an increase in CHO concentration. However, there was no decrease in the number of such embryos for calli transferred from IM with sucrose to MM with sucrose or fructose, as CHO increased from 3 or 4 to 6%, respectively (Table 2, Fig. 2B).

With respect to bipolar embryos, the significant I x M x C interaction (Table 1) was apparently due to a steady increase in the number of such embryos at 2 to 5%, 2 to 6% and 3 to 6%, for calli transferred from sucrose to fructose, sucrose to glucose and glucose to fructose, respectively; whereas, for most other calli, the number of bipolar embryos mainly increased as CHO increased from 2 to 3 or 4% and did not increase or decreased at 4 to 6% (Table 2, Fig. 2C). Calli transferred from IM with sucrose to MM with fructose at 5 and 6%, had the highest number of bipolar embryos (127 and 109/g callus, respectively). Calli transferred from IM with fructose or glucose to MM with fructose at concentration of 6%, also had a high number of bipolar embryos (99 and 83/g callus, respectively). On the other hand, calli transferred from IM with glucose to MM with glucose or fructose at a concentration of 2% gave rise to

the lowest number of bipolar embryos (26 and 33/g callus, respectively; Table 2, Fig. 2C).

No interactions were significant with respect to conversion rate of embryos to plantlets. However, CHO type in IM and MM, and CHO concentration individually affected the conversion rate (Table 1). Sucrose in IM gave rise to a higher conversion rate than glucose and fructose on the subsequent MM (4.8, 3.8 and 3.7 plantlets/g callus, respectively). Fructose and sucrose in MM gave rise to a higher conversion rate than glucose (4.8, 4.4 and 3.2 plantlets/g callus, respectively). The CHO concentrations of 5 and 6% gave the highest conversion rates (5.6 and 5.7 plantlets/g callus, respectively). These were significantly higher than that at CHO levels of 2 and 3% but not than that at 4% (1.9 and 3.5 and 4.4 plantlets/g callus, respectively). The combination of IM with sucrose and MM with fructose at 5 and 6%, resulted in the highest conversion rate (7.6 and 7.2 plantlets/g callus, respectively). Whereas the combination of IM and MM each with glucose at 2% had the lowest (0.8 plantlets/g callus; Table 2, Fig. 2D).

Carbohydrate influence on callus growth and number of embryo on IM with NAA

Fresh weight callus increase was highest on IM supplemented with glucose or sucrose, significantly higher at 3 and 4% (812-972%), than on IM containing the same fructose levels (527-604%; Fig. 3A). Fresh weight of callus

increased as glucose and sucrose levels increased from 2 to 3% (686 to 862% and 782 to 972%, respectively) and decreased at 4 to 6% (821 to 480% and 877 to 499%, respectively; Fig 3A). With respect to fructose, callus fresh weight was highest at 2% (616%), and gradually decreased at 3 to 6% (604 to 398%; Fig. 3A).

Glucose and sucrose promoted higher embryo formation than fructose. On IM with glucose callus was granular and mostly contained small (<2mm diam) dense and globular embryos, but their number was not as high as shown for calli on IM containing 2,4-D. Embryo number increased as glucose increased from 2 to 3% (282 to 340/g callus), and decreased at 4 and 6% (337 to 248/g callus; Fig. 3B). Sucrose induced formation of fewer embryos which were slightly larger and elongated than those formed on glucose. Embryo number increased as sucrose level increased from 2 to 3% (275 to 310/g) and decreased at 4 to 6% (309 to 242/g callus; Fig 3B). Similar to the 2,4-D results, fructose induced formation of large, swollen and vitrified embryos. Their number slightly decreased as fructose level increased from 2 to 6% (183 to 141/g callus; Fig. 3B).

Carbohydrate influence on callus growth and number of embryos on MM for calli derived from IM with NAA.

Calli fresh weight on MM consistently increased with an increase in CHO concentration (Table 3, Fig 4A). The interaction I x M x C was highly significant with respect to

callus growth and significant with respect to number of globular and bipolar embryos (Table 1).

The interaction in callus growth was mainly due to a significant increase in fresh weight of calli transferred from IM with glucose or fructose to MM with glucose, at 2 to 6% (779 to 2134% and 710 to 2114%), while calli from other IM to MM sequences had only a gradual or differential increase from 2 to 6% (e.g. 672 to 961%, for calli transferred from IM with sucrose to MM with fructose; Table 3, Fig 4A).

For most calli the number of globular embryos decreased as CHO concentration increased from 2 to 6%. Calli transferred from IM with fructose to MM with sucrose at 2%, had the highest number of globular embryos (130/g callus), and their number sharply decreased at 3% (90/g callus), and gradually decreased at 4 to 5% (93 to 68/g callus), remaining constant at 6% fructose (72/g callus; Table 3, Fig. 4B). Calli transferred from IM with glucose to MM with fructose, across all concentrations had the lowest formation of globular embryos as concentration increased from 2 to 6% (70 to 36/g callus; Table 3, Fig. 4B). Also, calli transferred from IM to MM each with fructose had a relatively low number of globular embryos at 2% (79/g callus), and the number remained relatively constant as CHO concentration increased from 2 to 5% (79 to 72/g callus), and decreased at 6% (51/g callus; Table 3, Fig. 4B). Calli transferred from IM with fructose to MM with glucose had a

similar pattern where embryo number slightly decreased at 2 to 5% (97 to 80/g callus) and decreased at 6% (55/g callus; Table 3, Fig. 4B). On the other hand, in calli transferred from IM to MM each with glucose, the number of globular embryos gradually decreased as CHO concentration increased from 2 to 4% (99 to 73/g callus), and did not decrease at 5% (72/g callus), but at 6% (55/g callus; Table 3, Fig. 4B).

With respect to bipolar embryos, the significant interaction I x M x C was mainly due to a marginal increase in number of embryos in calli transferred from IM to MM each with glucose as CHO levels increased from 2 to 6%, while calli in the other IM and MM CHO combinations had a higher and/or differential increase rate (Table 3, Fig 4C).

The CHO type in MM and CHO concentration had independent effects on conversion of embryos into plantlets and no interactions were significant (Table 1). Fructose in MM had a significantly higher conversion rate than glucose or sucrose (6.2, 3.9 and 4.9 plantlets/g callus). The CHO concentrations of 5 and 6% gave the highest conversion rate (7.4 and 7.2 plantlets/g callus).

These were significantly higher than that at 4% which was significantly higher than these at 3 and 2% (5.1, 3.4 and 1.8 plantlets/g callus, respectively). The combination of sucrose in IM and fructose in MM at 5% and glucose in IM and fructose in MM at 6% gave the highest conversion rate (10.2 and 9.8 plantlets/g callus, respectively) while the combination of glucose or fructose in IM and glucose in MM

at 2% resulted in the lowest conversion rate (0.6 plantlets/g callus; Table 3, Fig. 4D).

On MM with 2 and 3% glucose, most embryos remained small and globular, while at 4, 5 and 6%, many mature embryos consisted of a long radical and a shortened shoot (Fig. 5). On the other hand, on MM with 2-5% fructose embryos gave rise to plantlets with a well developed shoot and a shortened root, while on MM with 6% fructose many plantlets had an elongated root. On MM with 2% sucrose most embryos remained small and globular, whereas at 3-6% mature embryos gave rise to well developed plantlets.

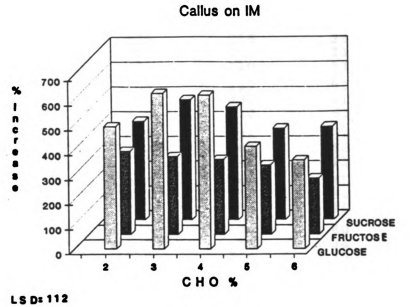
Table 1. Analysis of variance for callus increase and frequency of somatic embryos as influenced by sugar types and concentration in IM and MM. IM containing 2,4-D.

Source	D.F.	% Callus increase	Globular Embryos	Bipolar Embryos	Plantlets %
R	5	NS	NS	NS	NS
I	2	**	**	**	*
M	2	NS	*	**	**
I x M	4	**	**	**	NS
C	4	**	**	**	**
I x C	8	**	*	*	NS
M x C	8	**	NS	**	NS
IxMxC	16	**	**	**	NS

IM containing NAA.

Source	D.F.	% Callus Increase	Globular Embroys	Bipolar Embryos	Plantlets %
R	5	NS	NS	NS	NS
I	2	**	**	**	NS
M	2	**	*	**	**
I x M	4	**	**	**	NS
C	4	**	**	**	*
I x C	8	**	*	NS	NS
M x C	8	**	NS	NS	NS
IxMxC	16	**	**	*	NS

**, *, NS, significant at the 1%, 5%, or not significant, respectively.
I = Sugar type in IM, M = Sugar type in MM,
C = Sugar concentration.



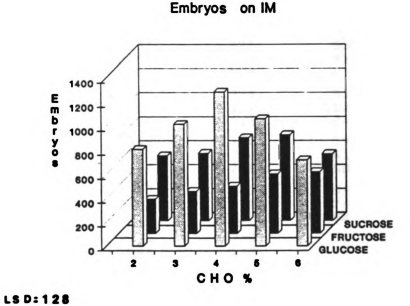


Fig 1: The effect of CHOs in IM containing 2,4-D (1.5ppm) on A) callus growth, and B) formation of small globular embryos after 4 weeks.

Table 2. Combined effect of sugar type and concentration in IM containing 2,4-D and MM on callus growth and frequencies of somatic embryos.

Sugar %	IM	MM	% Callus Increase	Globular (<3mm)	Bipolar (4-7mm)	Plantlets
	G	G	876	286	26	0.8
2	G	F	913	151	33	1.0
	G	S	804	220	53	1.8
	F	G	868	259	53	1.7
2	F	F	1095	237	61	2.0
	F	S	915	310	55	1.7
	S	G	639	438	33	2.3
2	S	F	1102	262	66	3.0
	S	S	797	245	71	3.0
	G	G	1316	245	41	3.2
3	G	F	1361	150	54	3.5
	G	S	1097	284	45	4.7
	F	G	1193	184	60	2.2
3	F	F	1172	155	86	3.5
	F	S	1914	216	73	3.2
	S	G	1114	326	41	3.7
3	S	F	1102	221	85	3.8
	S	S	1236	145	92	3.5
	G	G	1499	207	53	2.0
4	G	F	1270	157	46	4.0
	G	S	1461	263	61	2.8
	F	G	1963	225	56	2.5
4	F	F	2007	146	82	4.2
	F	S	2404	158	67	4.0
	S	G	1168	272	59	4.8
4	S	F	1263	124	112	6.0
	S	S	1358	144	94	5.2
	G	G	2341	170	39	2.5
5	G	F	1947	126	61	6.0
	G	S	1875	218	50	6.2
	F	G	2183	147	52	4.7
5	F	F	2453	129	74	6.3
	F	S	1914	122	64	6.0
	S	G	1731	185	61	5.0
5	S	F	1644	119	127	7.6
	S	S	1935	160	69	6.0
	G	G	2916	125	39	4.0
6	G	F	2062	88	83	7.0
	G	S	2027	156	50	6.8
	F	G	2779	155	74	3.8
6	F	F	2098	109	99	5.7
	F	S	2355	116	55	5.8
	S	G	2151	214	67	5.7
6	S	F	2000	121	109	7.2
	S	S	1887	148	79	5.3
	LSD =		380*	72.5*	21.3*	4.1*

**, *, NS, significant at the 1%, 5% levels, or not significant, respectively.
G = Glucose, F = Fructose, S = Sucrose.

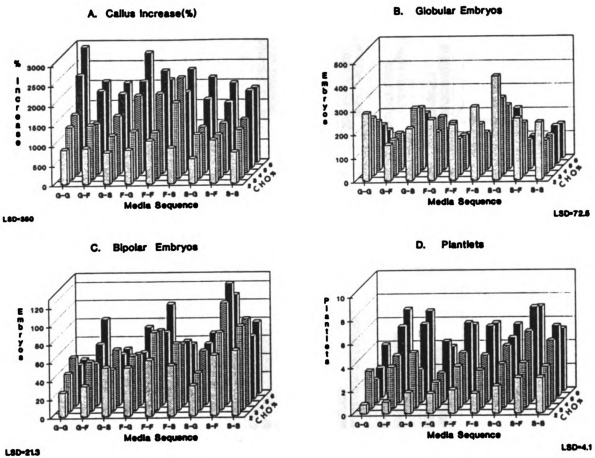
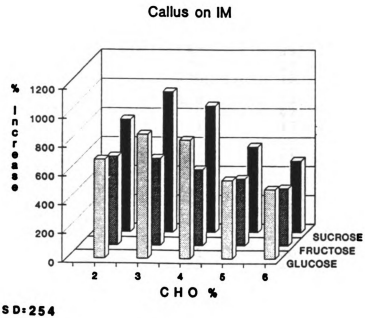


Fig 2: The combined effect of CHOs in IM containing 2,4-D and MM on A) callus increase, B) number of globular, C) bipolar embryos, and D) plantlets. G-glucose, F-fructose, S-sucrose.



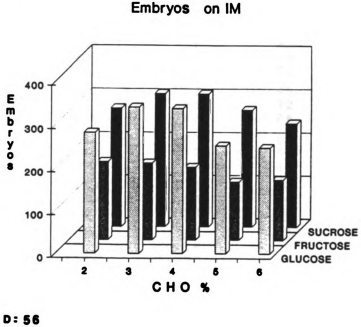


Fig 3: The effect of CHOs in IM containing NAA on A) callus increase, and B) number of embryos per gram callus. After 4 weeks.

Table 3. Combined effect of sugar type and concentration in IM containing NAA and MM on callus growth and frequencies of somatic embryos.

Sugar %	IM	MM	% Callus Increase	Globular (<3mm)	Bipolar (4-7mm)	Plantlets
	G	--> G	779	99	27	0.7
2	G	--> F	591	70	41	2.3
	G	--> S	689	105	36	1.3
	F	--> G	710	97	33	0.8
2	F	--> F	679	79	28	3.3
	F	--> S	743	130	33	0.7
	S	--> G	611	128	33	1.8
2	S	--> F	672	77	42	3.0
	S	--> S	652	104	35	2.5
	G	--> G	900	87	35	2.2
3	G	--> F	951	55	51	3.3
	G	--> S	866	108	49	2.5
	F	--> G	1063	95	47	3.5
3	F	--> F	783	79	40	5.2
	F	--> S	886	90	38	4.2
	S	--> G	917	99	38	2.0
3	S	--> F	786	81	60	4.5
	S	--> S	833	82	42	3.5
	G	--> G	1211	73	33	3.7
4	G	--> F	1052	58	49	4.8
	G	--> S	1249	80	47	4.5
	F	--> G	1270	75	57	4.2
4	F	--> F	1024	68	39	5.8
	F	--> S	1031	93	46	5.2
	S	--> G	1063	121	65	5.3
4	S	--> F	909	51	58	7.5
	S	--> S	922	86	55	5.2
	G	--> G	1452	72	43	4.2
5	G	--> F	1142	54	72	7.7
	G	--> S	1665	89	66	7.3
	F	--> G	1271	80	69	6.2
5	F	--> F	1440	72	56	8.5
	F	--> S	1197	68	44	8.7
	S	--> G	1129	78	63	7.2
5	S	--> F	949	54	76	10.2
	S	--> S	1287	58	58	6.5
	G	--> G	2134	55	41	4.8
6	G	--> F	1509	36	70	9.8
	G	--> S	1778	70	71	7.8
	F	--> G	2114	55	65	4.7
6	F	--> F	1543	51	64	8.5
	F	--> S	1199	72	58	7.5
	S	--> G	1113	74	78	6.5
6	S	--> F	961	43	74	9.3
	S	--> S	1361	63	57	6.0
	LSD	=	332**	21.7**	13*	4.6**

**, *, NS, significant at the 1%, 5% levels, or not significant, respectively .
G = Glucose, F = Fructose, S = Sucrose.

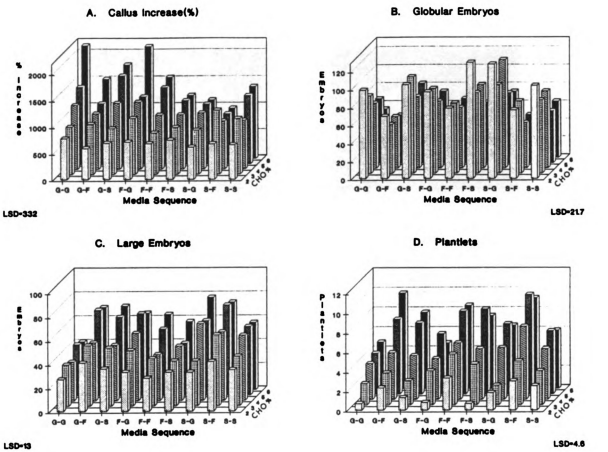


Fig 4: The combined effect of CHO's in IM, containing NAA, and MM, on A) callus increase, B) number of globular and C) bipolar embryos, and D) plantlets.

Fig. 5. Typical mature embryos and plantlets formed on MM containing 5% glucose (G), fructose (F) or sucrose (S). (x 18).

DISCUSSION

Callus growth on IM was reduced as glucose or sucrose levels increased from 3 or 4 to 6%, and as fructose levels increased from 2 to 6%, whereas increased callus growth was observed on the subsequent MM with equivalent osmotic potentials but a low auxin level (NAA 0.01ppm). Additionally, glucose and sucrose in IM supported a higher callus growth than fructose, while in the subsequent MM this effect was not as pronounced and callus growth on fructose at 2-6% resembled that of callus on MM with glucose or sucrose of the same levels. These differences in callus growth on IM and MM may be explained either by a slower uptake and/or metabolism of CHOs, mainly fructose, in the presence of auxin or by slow adaptation of the callus to grow on fructose following induction with auxin (Verma and Dugall, 1977).

It is likely that in the presence of auxin, fructose uptake occurred at the same rate as that of glucose and sucrose, but may have been metabolized at a lower rate. This deduction is based on observations of IM and MM containing fructose where embryos formed in a low frequency but were large and vitrified as compared with those on glucose and sucrose. The increased size and vitrification may have resulted from accumulation of fructose which constituted a high cellular osmoticum that led to increased diffusion of water from the medium to the embryoid tissue. Wright and Northcote (1972) suggested that various CHOs may

be metabolized through a common intermediate. However, the rate of uptake and/or initial metabolism of CHOs to the common intermediate would control the rate of growth and embryogenesis.

Maretzki et al. (1974) concluded that sucrose and glucose are the best carbon sources for growth of cultures, but other CHO types may substitute depending on the plant species and the tissue used. It was demonstrated herein that auxin type and level and/or developmental stage of the culture also influenced CHO utilization. On IM with 2,4-D and glucose, a vigorous callus with a fine granular texture and numerous small globular embryos occurred. This suggests that 2,4-D increases glucose metabolism, where glucose in turn enhanced the formation of numerous globular embryos. However, in the presence of NAA the effect of glucose in IM was not as pronounced on callus growth and there was no difference between calli on IM with glucose or sucrose with respect to frequency of small globular embryos formed. Although glucose enhanced embryo formation and growth, it was not the most effective carbohydrate source for embryo differentiation. Glucose enhanced the formation of secondary (small globular) embryos on MM, promoted the embryos to form a large radical, but suppressed shoot growth. On the other hand, fructose, which promoted embryos to form shoots, did not prove to be the best CHO sources for callus, root and embryo growth. Kononowicz and Janick (1984) showed that glucose blocked the maturation of cacao embryos, sucrose

supported it and fructose had an intermediate effect. The striking differences observed in embryo frequency and morphology may have resulted from an imbalance in CHOs used for cell metabolism and between those needed for the formation of major cell components (Maretzki et al. 1974) and/or between these two needs and those used for osmoticum (Lapena et al. 1988). In a preliminary experiment we deduced that sorbitol could not be metabolized by asparagus tissue cultures. However, the substitution of sucrose with sorbitol of the same molarity increased the number of regenerated shoots from 1.3 to 4.6 shoots per gram callus (appendix). Work with tobacco (Brown et al. 1979) and rice callus cultures (Kishor, 1987) showed that part of the supplemented CHO played an osmoregulatory role during shoot formation. This may indicate that fructose, which is metabolized at a slower rate than glucose and sucrose, may accumulate in the cytoplasm and have an osmoregulatory role similar to that of sorbitol, which enhanced shoot growth in the developing embryo.

Although the interaction between CHO type in IM x CHO in MM x CHO concentration was significant with respect to callus growth and embryo formation, it was not significant with respect to the rate that embryos converted into plantlets. However, CHO type in IM containing 2,4-D and in MM, and CHO concentration had a significant effect on conversion rate. These indicate that the induction and maturation stages may have differential requirements for CHO

type and concentration. Kononowicz and Janick (1984) concluded that successful maturation of zygotic embryos of Theobroma cacao required a distinct shift from the growth phase to the accumulation phase of embryo development, and this shift was greatly affected by carbon source. However, when NAA was used, CHO type in IM did not have a significant effect, whereas CHO type in MM affected the conversion rate. Thus, auxin type used in IM appears to nullify the differential need for CHOs to maximize conversion.

As mentioned previously, fructose in MM increased conversion rate by promoting embryos to form shoots, while glucose suppressed shoots but enhanced root growth. Interestingly, sucrose which contains both hexoses and it promoted plantlets with shoot and root growth occurring simultaneously. Kikuta and Okazawa (1984) found that mannitol, sorbitol and inositol supported shoot bud formation in potato tuber tissue, whereas, sucrose promoted only root formation. These researchers suggested that CHO source may influence the type of organ differentiated.

The CHO concentration in both experiments had a marked effect on callus, embryo frequency, differentiation and conversion into plantlets, indicating that levels of 5-6% are optimal for asparagus embryo growth and germination.

Lazzeri et al. (1988) demonstrated a significant interaction between sucrose and auxin in soybean somatic embryogensis. High sucrose levels (6-12%) also promoted the formation of embryogenic callus in maize (Lu et al. 1983).

The younger the zygotic embryos, the higher the CHO levels required for their development in-vitro (Yeung et al. 1981). On the other hand, low sucrose levels stimulated the growth of Theobroma cacao somatic embryos (Konowicz and Janick, 1984). In our study, CHO type was different but the levels remained constant during induction and maturation of embryos. However, another study (Levi and Sink, 1990) indicated that placement of globular embryos which formed in suspension on solidified MM with high CHO levels (4-10%) for two weeks followed by transfer of mature embryos to MM with low CHO level (2%), significantly enhanced embryo conversion into plantlets. It was found here that CHO concentrations of 5-6% are optimal for both early and late stages of somatic embryogensis. It is however apparent that successful embryogenesis may require high CHO levels during the induction stage followed by gradual transfer of embryos to MM with lower CHO levels for further maturation and development to plantlets (Levi and Sink 1990).

The present study demonstrated that variations in CHO source and concentration have major effects on the morphogenesis and frequency of asparagus somatic embryos. Furthermore, the type of CHO used at the different developmental stages is important in normalizing embryogenesis. Placement of callus on IM (containing 1.5ppm NAA), with sucrose and MM with fructose at 5-6% was optimal for embryo development and conversion to plantlets.

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SECTION IV

PRODUCTION OF ASPARAGUS SOMATIC EMBRYOS THROUGH SUSPENSION CULTURE

ABSTRACT

Methods were studied to enhance the frequency of asparagus somatic embryos produced in suspension culture and their subsequent rate of conversion into plantlets on solidified maturation media (MM). Stalk-derived callus subcultured twice on Murashige and Skoog (1962) salts and vitamins (MS) medium + NAA (0.1ppm) and 2iP (0.3ppm), was used to establish suspension cultures. Six out of fifteen suspensions, induced with NAA (10-20ppm) were found to have a high embryogenic capacity. Such embryogenic suspensions consisted primarily of single, elongated cells (about 90%), organized cell clusters (2571/ml) and globular translucence embryos (32/ml) which developed into plantlets within two to four weeks on MM of MS + NAA (0.1ppm) and 2iP (0.2ppm). Using embryos derived from suspension line 10-1 (MS + 15ppm NAA), it was found that a low carbohydrate (CHO) level (2%) in MM increased the formation of small secondary globular embryos (251-258/g callus). In contrast, higher CHO levels (6-10%) of fructose or sucrose, reduced the occurrence of secondary embryos (30-85/g callus) while simultaneously promoting the growth and maturation of individual embryos; 3.6-8.5 higher than 2% CHO. Furthermore, transfer of globular embryos from suspension to MM with high CHO levels (4-10%) for two weeks followed by transfer to MM with a low

CHO level (2%) resulted in a 2-4 fold higher germination of embryos into plantlets in comparison to those that were subcultured at CHO levels of 4-10%. Mature plantlets placed singly in culture tubes containing MM + glucose (2%) and ABA (0.7ppm) developed a crown with an extensive root system and limited callus formation at the crown. Such crowns had a higher survival rate (82%) when transferred to planting medium, as compared to those grown on MM with 2% sucrose (52%).

INTRODUCTION

Asparagus officinalis L. was one of the first monocot species to regenerate somatic embryos through suspension cultures (Wilmar and Hellendoorn, 1968; Steward and Mapes, 1971). The former authors reported that hypocotyl-derived callus formed a large number of globular embryos when placed on solidified Linsmaier and Skoog (1965) medium (LS) + 2,4-D (1.0ppm) and kinetin (0.3ppm), and in liquid suspension of the same composition both gave rise to mature plantlets. Steward and Mapes (1971) reported the formation of somatic embryos from asparagus stem segments through a sequence of callus initiation in White's liquid medium + coconut milk (CM) + NAA followed by replacement of NAA with 2,4-D, and induction of embryoids in high salt LS + NAA. Root and shoot primordia were promoted by transfer of embryoids to LS + CM + IAA. Neither report quantified the embryogenic responses obtained nor mentioned the efficiency in conversion of embryos to plantlets. Our attempts to use

these protocols with a different genotype yielded mostly abnormal embryogenic structures and only a few normal embryos and plantlets.

In related studies (Levi and Sink, 1989a), callus from in vitro established crowns and lateral bud explants were both found to be the optimum tissue for initiating embryogenesis. NAA alone (1-10ppm) was sufficient for the induction of asparagus somatic embryos with the capacity to convert into plantlets. In addition, CHO source and concentration in induction medium (IM) and MM were found to affect both embryo frequency and morphogenesis (Levi and Sink, 1989b).

The objective of this study was to determine the possibility of large scale propagation of elite, mature asparagus crowns through embryogenic suspension cultures.

MATERIALS AND METHODS

Stalks (2-6mm diam) of mature asparagus crown M.S.U. male 88-10 were collected, sliced into 5-8 cm sections (4-7 internodes), and surface sterilized for 30 min in aqueous sodium hypochlorite (1.5% v:v) with 2 drops of Tween-20. Stalks were thoroughly rinsed with 3 changes of sterile distilled water. To initiate callus, 3-4 stalks were placed horizontally in 100 x 15mm Petri dishes containing 25 ml of callus initiation medium of MS + NAA (0.1ppm) + 2iP (0.03ppm), and containing 3% sucrose and 0.9% Difco agar with the pH was adjusted to 5.9 prior to 20 min autoclaving (Levi and Sink, 1989a). The dishes were placed at 26C under

16h light of 16uEm^{-2} sec^{-1} from cool white fluorescent bulbs (Sylvania, GTE). Primary callus formed within 4-8 weeks at the periphery of the lateral meristems on the stalks. This callus was removed and subcultured twice at 4 week intervals.

Subsequently, 0.5 gram of callus was transferred to each of twelve 250 ml Erlenmeyer flasks each containing 40 ml of liquid MS initiation medium. The flasks were placed on a gyratory shaker at 150 rpm in the same environment mentioned above. Three weeks later, the suspensions were sieved through a 600u mesh screen. The filtrates were collected and centrifuged (125 g; 5 min) and the pelleted cells, 0.1 gram, were transferred to each 250 ml Erlenmeyer flask containing 40 ml fresh liquid MS + NAA at various levels (0.1, 1, 3, 10, 15, or 20ppm); five replicate flasks per level. After 3 weeks of further culture, all suspensions were filtered through a 600u mesh screen and the large cell clumps (about 5mm+ diam) were removed and discarded. The remaining cell aggregates and embryos of similar size (0.5-3mm diam) were washed once with MS basal medium. Five to eight tissue samples (0.1g about 250-350 small embryos and cell aggregates/g tissue) were harvested from each suspension and placed in each 15x100m Petri dish containing 25ml MM of MS + NAA (0.1ppm) and 2iP (0.2ppm). The dishes were placed at 26C under 16h of light at 16uEm^{-2} sec^{-1} from cool white fluorescent bulbs. The remaining sieved cells from each suspension were collected

by centrifugation as described above, and the pelleted cells (about 0.1g) were resuspended in 40 ml fresh medium to maintain the culture line. Two and four weeks later the tissue samples on MM were evaluated for embryo and plantlet frequency and morphology.

Donor suspensions that gave rise to a high frequency of abnormal structures during the evaluation were discarded, while those producing normal bipolar embryos and plantlets were retained by subculturing every 3 weeks. Suspensions were also evaluated for embryogenic potential using the following criteria 1) frequency of single, elongated vacuolated cells, 2) organized clusters of cells with enriched cytoplasm, 3) proembryogenic masses consisting of elongated vacuolated cells and a few (about 2-8) globular embryos at early developmental stages (about 8-64 cells), 4) translucent globular embryos (0.2-4mm diam) with a smooth spherical profile.

To further characterize the differences between the nonembryogenic (initial) and embryogenic (induced with NAA) the number of single cells, cell clusters, cell clumps, roots and embryos were determined in 8-12 samples (1ml) withdrawn randomly from cultures in liquid MS + 0.1, 3 and 15ppm NAA, and at a cell density of 10^6/ml. An analysis of variance (AOV) was performed for the traits in each suspension.

Five selected embryonic cell lines (three, two and one which were formed in liquid MS containing 10,15, or 20ppm

NAA, respectively) were maintained for 4-6 months by the routine protocol, each in 2-4 250 ml flasks containing 40 ml liquid MS with the same starting NAA level. A sixth embryonic cell line designated 10-1 which was formed in MS + NAA (15ppm) was maintained for over ten months in 12-18 flasks containing 40 ml of this medium. During this time line 10-1 was used for the study of the effect of CHO type and concentration in MM on embryo development and rate of embryo conversion to plantlets.

Globular embryos and small cell aggregates of similar size (0.5-3mm diam) in line 10-1, were harvested three weeks after sieving and subculturing as described above. One gram samples (about 150-300 embryos and small cell aggregates) were transferred to each 100 x 15mm Petri dish (6 per CHO treatment) containing 25 ml of MM with glucose, fructose or sucrose at various levels (2, 4, 6, 8, and 10%). The dishes were placed in the same environment used for callus initiation. After two weeks on MM, embryos were either subcultured or transferred to MM with a lower level of agar (0.8%) and the same type of CHO at 2%, i.e. a sequence of 4-->2%, 6-->2%, 8-->2%, 10-->2%, (Table 2). Two and four weeks later, respectively, the number of embryos and plantlets per gram tissue was determined by withdrawing randomly a 2-3g tissue sample (callus and embryos) from each Petri dish and counting the embryos using a dissecting microscope. Small spherical embryos (<3mm diam) were classified as globular, and large elongated embryos (about

4-7mm) as bipolar. Elongated embryos of various sizes (about 2-7mm) but with a whitish, opaque appearance and a distinct shoot primordium and a root were classified as mature embryos. Embryos that had developed a green shoot and hairy elongated root were classified as plantlets. The conversion rate of embryos to plantlets was determined as: % plantlets = plantlets /plantlets + mature embryos x 100. This experiment was conducted 2 factor factorial with 3 CHO types x 9 sequences x 6 replications (each Petri dish) in a randomized complete block design (RCBD). Mean separation was by LSD (Steele and Torrie, 1980).

In a third study, individual plantlets, derived from line 10-1 somatic embryos on MM + fructose (8%), were transferred singly to 15 x 2.5 cm glass culture tubes each containing 20ml MM + sucrose or glucose (2%) and ABA (0, 0.1, 0.7, 2, or 3ppm). Six weeks later, crowns were removed from culture and visually evaluated for fern and root growth (50-150 crowns/treatment) prior to their transfer to high humidity conditions in a planting medium of soil, sand, and Bacto Mix (Michigan Peat Co. Houston, TX) (1:1:1, v:v:v), or to Bacto Mix: Polypropylene beads (Northern Petrochemicals, Des Plaines, IL) 3:1 (v:v).

RESULTS

The initial suspension cultures in liquid MS + (ppm): NAA (0.1) and 2iP (0.03) were yellowish-green and contained many large and unorganized cell clumps. Three to four weeks after the first sieving and subculture, suspensions

at a cell density of 10^6/ml consisted of about 71% spherical cytoplasmically rich cells, and 29% elongated vacuolated cells. Most of these cells were grouped in unorganized clusters (about 5070 clusters/ml), while only a few clusters (about 600/ml), had organized cell division. Large unorganized cell clumps (0.5-4mm diam) and roots (0.1-8mm), (4.1/ml and 3.3/ml, respectively) and only a few globular (0.2-2mm diam) and elongated embryos (0.5-4mm), (2.3 and 3.4/ml, respectively; Table 1) were present.

In contrast to the initial suspension cultures, suspensions in MS + NAA (1 or 3ppm) consisted of many free cells and globular and elongated embryos. Three to four weeks after two subcultures in liquid MS + NAA (3ppm), suspension cultures at a cell density of 10^6/ml consisted of many (72%) single, elongated vacuolated cells and a lower frequency (28%) of large spherical cells. Such suspensions were also composed of many (1863/ml) organized clusters of spherical and richly cytoplasmic cells, and a smaller portion (710/ml) of unorganized clusters with elongated cells which divided randomly (Table 1). Large (0.5-4mm diam) unorganized cell clumps (0.5-4mm);1.6/ml, fused roots, (0.1-5mm); 2.9/ml, and globular, (0.2-3mm diam); 14/ml, and elongated, (0.2-4mm) embryos; 8.7/ml were also present (Table 1). The NAA (1 and 3ppm) suspensions gave rise to many abnormal embryos and fused roots (about 87-165 and 40-85/g callus, respectively) and to only a few normal embryos

and mature plantlets (about 32-70 and 1-3/g callus, respectively) within 4 weeks on MM.

The suspension cultures of MS + NAA 10, 15, or 20ppm had a homogeneous fine cell texture and consisted mainly of single elongated vacuolated cells, organized clusters (pro-embryogenic mass) of enriched cytoplasmic cells, and globular translucent embryos each attached to a few suspensor cells. These suspensions were devoid of organized structures such as roots and meristemoids. However, only two, three and one out of five suspensions at each level, respectively, gave rise to a high frequency of bipolar mature embryos (34-70/g callus) and vigorous plantlets (7-24/g callus) on MM. These cultures were classified as highly embryogenic, and were maintained over a period of ten months. Suspensions in liquid MS + NAA (15ppm) and the same cell density used above, consisted of many free cells. Most of them, about 90%, were elongated and crescent-shaped and the rest, about 10%, were spherical cytoplasmically rich cells containing many starch grains. Additionally, many (2571/ml) prembryogenic clusters of cells which divided longitudinally and transversally, and only a few unorganized clusters (173/ml) were present (Table 1). Such suspensions contained many (31.6/ml) single globular translucent embryos at early and late developmental stages (0.2-3mm diam) and a few (about 1/ml) bipolar embryos (0.2-5mm) (Table 1).

The interaction CHO type (T) x concentration sequence (C) on MM was significant with respect to number of

globular, bipolar and mature embryos, but not for conversion rate of embryos into plantlets (Table 2). Low CHO level (2%) in MM promoted the secondary formation of small globular embryos, and gave rise to only a few bipolar and mature embryos and plantlets (Table 3, Fig. 1A). The frequency of secondary globular embryos was significantly higher on glucose or sucrose than on fructose at each 2% (258, 251 and 165/g callus, respectively). However, there were no differences in frequency of small globular embryos between sucrose and fructose at the higher levels of 4 to 10%, except between these two and glucose at the 4% level (89, 103 and 153/g callus, respectively). Additionally, there was a significant difference between glucose and fructose at the level of 6% (115 and 61 globular embryos/g callus, respectively), and for the concentration sequence of 8-->2% (110 and 72/g callus, respectively). Additionally, the number of globular embryos was significantly higher on MM with glucose than on MM with sucrose for the sequence of 6-->2% (126 and 90/g callus, respectively; Table 3, Fig 1A). The transfer of embryos from high (4-10%) to low CHO concentration (2%) did not lead to a significant change in the frequency of secondary globular embryos (Table 3, Fig 1A).

High CHO levels (4-10%) inhibited the formation of globular embryos and simultaneously promoted growth of bipolar embryos with an average size that increased with an increase in CHO level (Table 3, Fig. 1B). High glucose or

sucrose levels (8-10%) in MM frequently gave rise to unbalanced embryo development where an excessively large radical was formed but the cotyledon and primary shoot each remained small. Fructose of the same concentrations frequently gave rise to embryos with a developed shoot portion and a small radical.

The significant T x C interaction for frequency of bipolar embryos was apparently due to a differential increase in the frequency of such embryos as the glucose concentration sequence changed from 6-->2% to 8-->2% (28.2 and 59.3 bipolar embryos/g callus, respectively), while there was no difference between these treatments for fructose and sucrose (42-45.3/g callus) (Table 3, Fig. 1B). Additionally, the number of bipolar embryos increased at glucose levels of 8 to 10% (52.7 to 62.8/g callus), while it decreased at the same levels of sucrose (56.3 to 41.3/g callus; Table 3, Fig. 1B).

Only a few mature embryos occurred on MM with a low CHO level (2%); 8.8-10.7/g callus. Their number increased at higher CHO levels (4-8%); 29.5-56/g callus, and decreased at 10% (19.3-32.2/g callus; Table 3, Fig. 1C).

The significant T x C interaction for mature embryos was due to an abrupt increase in the frequency of such embryos at sucrose levels from 2 to 4% (10.5 to 50.5/g, respectively), while on MM with glucose at the same levels the increase was not as pronounced (8.8 to 29.5/g callus; Table 3, Fig. 1C). The transfer of embryos from MM with

high CHO levels (4-8%) to MM with a low CHO level did not result in a significant change in the number of mature embryos as compared to those subcultured on MM with a constant CHO concentration (Table 3, Fig. 1C). Except, a significant increase in the frequency of such embryos at the glucose sequence of 8-->2% (71/g callus; Table 3, Fig. 1C). As compared to subculture on MM with a constant level of 10% glucose or sucrose, the concentration sequence of 10-->2% had a significant increase in the number of mature embryos (from 19.3 to 34.8 and from 27.2 to 48.5, respectively). There was no change in the number of such embryos on MM with fructose (32.2 and 35.8/g callus; Table 3, and Fig. 1C).

The interaction T x C was not significant with respect to number of plantlets, but highly significant effect were noted for T and C alone (Table 2). Carbohydrate levels of 4-10% promoted the growth and development of individual embryos giving rise to a higher frequency of plantlets (2.8-13.8/g callus) than a CHO level of 2% (0.8-1.7/g callus). Furthermore, the transfer of embryos from high (4-10%) to a low CHO (2%) significantly increased their conversion into plantlets by 1.9-4.3 fold (Table 3, Fig. 1D). Sucrose and fructose generally promoted a conversion rate that was significantly higher than that of glucose (an average of 14.7; 22.9%, 12.9; 24.5% and 9.8; 19.8% plantlets/g callus, respectively). The fructose and sucrose concentration sequences of 6-->2% and 8-->2% were optimal for the

conversion of large and vigorous plantlets; 23-25.2/g callus (about 38.6-51.2%). Continued subculture of embryos on MM with CHO concentrations of 2-6%; at two-three week intervals, enhanced their development and conversion into normal plantlets in frequencies up to 68-81% within ten weeks on MM. A prolonged culture (4-10 weeks) on MM with high CHO levels (mainly on 8-10% glucose or sucrose) resulted in abnormal development of embryos and plantlets which had an enlarged singular thick root and a short or undeveloped shoot. Such plantlets had a poor survival following transfer to planting medium. The transfer of embryos from high to low CHO concentration not only increased the conversion rate, but also resulted in plantlets with thinner roots.

Mature plantlets transferred singly to culture tubes which contained MM with 2% sucrose developed a crown within 3-4 weeks. Callus frequently proliferated around the crown base and prevented the establishment of a well developed root system which resulted in a low survival rate (52-60%) in the planting medium. However, plantlets transferred to tubes containing MM + glucose (2%) developed a crown with an extensive root system within 3-5 weeks, while a moderate reduction in callus formation occurred. Furthermore, the addition of ABA, 0.7-2ppm, to MM with glucose (2%) further reduced callus formation and enhanced root development (Table 4, Fig. 2E). Crowns grown on MM with 2% glucose and 0.7ppm ABA had a higher survival rate (82%) than those on MM

with 2% sucrose and devoid of ABA (51%) when transferred to planting medium (Table 4).

Table 1. Mean and standard deviation of number of single cells, cell clusters and embryos as influenced by NAA level in 4 week old suspension cultures, at a final cell density of 10^6/ml.

	NAA (ppm)		
	0.1	3	15
Single cells			
% Elongated	30.1 ± 7.5	72.2 ± 7.7	90.6 ± 6.9
% Spherical	71.4 ± 12.4	34.4 ± 9.6	11.0 ± 6.2
Clusters			
Unorganized	5078 ± 1781	710 ± 479	173 ± 85
Organized	600 ± 341	1863 ± 497	2571 ± 735
Embryos			
Globular	2.3 ± 1.9	14.0 ± 4.6	31.6 ± 6
Elongated	3.4 ± 2.3	8.7 ± 2.7	1.0 ± 0.7

Table 2. Analysis of variance for number of embryos and plantlets as influenced by CHO type (T) and concentration sequence (C) in MM.

Source	DF	Globular (<3mm)	Bipolar (4mm+)	Mature embryos	Plantlets
R	5	NS	NS	NS	NS
C	8	**	**	**	**
T	2	**	NS	*	**
C x T	16	**	**	**	NS

**, *, NS, significant at 1%, 5% levels, or not significant, respectively.

Table 3. Influence of sugar type and concentration sequence in MM on number of somatic embryos and plantlets per gram callus.

CHO concn.(%)	CHO type	Globular (<3mm)	Bipolar (4-7mm)	Mature Embryos	Plantlets
	G	258.0	2.5	8.8	1.0
2-->2	F	164.7	4.7	10.7	0.8
	S	250.8	3.8	10.5	1.6
	G	152.5	25.2	29.5	5.8
4-->4	F	89.0	34.3	41.7	8.0
	S	102.5	29.3	50.5	9.0
	G	114.8	45.5	42.7	7.5
6-->6	F	61.0	59.0	55.8	9.5
	S	85.0	38.2	56.0	12.5
	G	79.2	52.7	42.3	6.0
8-->8	F	46.3	56.2	43.2	9.7
	S	62.3	56.3	48.0	11.8
	G	47.2	62.8	19.3	2.8
10-->10	F	38.5	52.5	32.2	5.5
	S	30.0	41.3	27.2	8.2
	G	114.2	26.3	36.0	12.3
4-->2	F	99.8	41.8	49.5	18.2
	S	97.3	44.8	61.2	21.8
	G	126.0	28.2	45.3	22.3
6-->2	F	93.2	43.5	55.7	24.1
	S	89.8	45.2	56.2	23.0
	G	110.0	59.3	71.0	19.3
8-->2	F	71.8	45.3	53.3	24.9
	S	76.2	42.0	54.7	27.0
	G	66.1	26.5	34.8	12.5
10-->2	F	50.5	39.8	35.8	16.6
	S	67.3	34.0	48.5	16.1
LSD =	(1%)	35.2	16.1	14.8	8.7

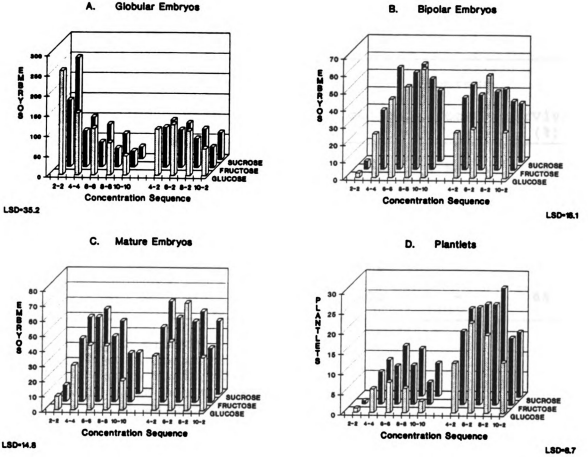


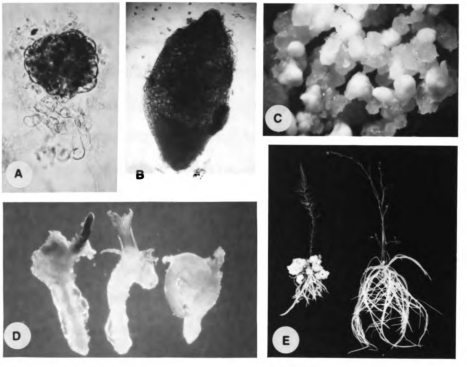
Fig. 1. Influence of carbohydrate type and concentration sequence in MM on number of A) globular, B) bipolar and C) mature embryos, and D) plantlets.

Table 4. Influence of CHO type and ABA level in MM on formation of roots and callus by plantlets grown in culture tubes, and survival rate of crowns two weeks after their transfer to planting medium.

	Glucose			Sucrose		
ABA (ppm)	callus*	root	survival (%)	callus	roots	survival (%)
0	1-3g	++	71	2-4g	++	52
0.1	1-3g	++	70	2-4g	+	60
0.7	<1g	+++	82	1-2g	+++	71
2	<0.5g	+++	74	<1g	++	73
3	<0.5g	++	69	<1g	+	66

0 - no root growth
+ - 1-2 main roots (2-5cm each)
++ - 3-5 main roots (2-8cm each)
+++ - 6-10 main roots (3-15 cm each)
* callus fresh weight per crown.

Fig. 2. A) Embryo in early globular stage in liquid suspension; MS + NAA (15ppm). (x 375). B) embryo in early bipolar stage after three days on MM. (x 52). C) Mature embryos after two weeks on MM with 6% sucrose (x 22.5) , and D) plantlets after additional two weeks on MM with 2% sucrose. (x 8.5). E) (left) crown developed on MM with 2% sucrose having callus at the shoot base and the forming roots, (right) a crown developed on MM with 2% glucose and ABA (0.7ppm) with a large root system and no callus (x 0.4).


A
B
C
D
E

DISCUSSION

A low level of NAA (0.1ppm) and 2iP (0.03ppm) in liquid MS were sufficient for asparagus cell suspension cultures to sporadically form embryos. These embryos, which frequently germinated into plantlets, may have originated from pre-embryogenic determined cells (Ammirato, 1986; Sharpe et al. 1980, 1982). In contrast, the large number of abnormal embryos formed in suspensions induced with NAA (1 and 3ppm) may have originated from nonembryogenic cells which had only a partial embryogenic capacity, due to induction with such levels of NAA. Higher levels of NAA (10-20ppm) may have promoted such cells to undergo complete embryogenic organization.

High NAA levels (10-20ppm) also induced a high frequency of asparagus somatic embryos in suspension and increased the capacity of such embryos to develop normally into plantlets on MM. Still, only six out of fifteen suspension cultures gave rise to cell lines that maintained a high embryogenic capacity. Ferriera and Handro (1988) reported that 2,4-D levels higher than 50mg/L were necessary for the induction of somatic embryos from zygotic embryos of Euterpe edulis Mart. (palmae). This observation indicates that auxins at high concentrations may be required for successful somatic embryogensis in some monocots. The rapid formation of a suspension with a fine cell texture which consisted of many single elongated cells enriched with cytoplasm and many starch grains, in addition to many

translucent embryos at the early and late globular stage, at high NAA levels, were typical of highly embryonic cell lines. Selection for such cell characteristics was essential to success in both obtaining and maintaining the embryogenic lines. The prolonged maintenance of embryogenic lines in suspension with high NAA levels also produced a high degree of synchrony in the formation of globular embryos of similar stage and size.

The CHO type and concentration were found to be important for embryo development while simultaneously inhibiting the formation of secondary embryos. The level of CHO in MM was also critical for increasing the conversion rate of embryos into plantlets and detrmining the type of plantlet formed. Carbohydrates at 6% in MM were optimal for normal embryo development and conversion into a vigorous plantlet; whereas, higher levels (8-10%) led to development of large embryos, but with an irregular shape; having an excessively large root and a small shoot. On the other hand, low CHO levels (2%) resulted in the formation of friable callus with numerous small globular embryos which may have been derived from pre-embryonic determined cells (Sharpe et al., 1980, 1982).

The results observed here are consistent with our previous finding (Levi and Sink, 1990b) that conversion rate was significantly higher at CHO levels of 5-6% than at 2-3%. Moreover, the transfer of embryos from MM with high (4-10%) to MM with a low CHO level (2%) significantly

enhanced the conversion of embryos into plantlets. Thus, indicating that asparagus somatic embryos may require high levels of CHO during their maturation stage and lower levels for subsequent germination.

In addition to their role as an energy and C source, CHOs are involved in regulating the osmotic potential of plant cells in vitro (Thompson and Thorpe 1987). Higher CHO levels in the medium during embryo development and maturation increased the osmotic potential in the embryogenic cells (Brown et al., 1979; Brown and Thorpe, 1980) and transfer of such embryos to a medium with a lower CHO level (a higher water potential) enhanced the uptake of water and subsequent germination. This pattern resembles the dehydration process where reserves are accumulated and the osmotic potential increases prior to the developmental arrest in the maturing seed embryo, whereas, subsequent imbibition initiates germination. Preliminary attempts to dehydrate embryos by transferring them from MM with low sucrose levels to higher levels (2-->3%, 3-->4%, 4-->6% etc.) frequently resulted in osmotic shock where mainly bipolar mature embryos turned brown and/or produced anthocyanin and grew slowly and/or abnormally (data not presented). This deterioration indicates that it is critical to start the dehydration process when the embryos are at the start of maturation stage. This is by transferring them from suspension (IM) to solidified MM containing a CHO at a level which increases embryo osmotic

potential. Finkelstein and Crouch (1986) indicated that low water potential is highly important in regulating the transition to developmental arrest during maturation of rapeseed embryos. Misra et al. (1984) reported that premature drying followed by dehydration of Phaseolus vulgaris L. or castor bean embryos, resulted in a shift in embryos developmental potential from embryonic to germinative growth reflected by patterns of protein synthesis. High osmotic potential was favored for embryonic differentiation (Granatek and Cockerline 1978; Lu et al., 1983), but may inhibit embryo germination (Lu et al., 1983) and growth of organs formed in vitro (Brown et al., 1979). Lazzeri et al. (1988) reported that the germination ability of soybean somatic embryos appeared to be influenced by sucrose concentration where embryos on 0.5% sucrose had the highest germination frequencies, while those on 4% sucrose had the lowest.

The higher conversion rate of embryos on MM with sucrose and fructose than with glucose was mainly due to development of embryos with a larger shoot primordia on MM, while glucose, mainly at 6-10%, induced embryos with a large root and an undeveloped shoot. Additionally, glucose (2-4%) adversely promoted the formation of secondary embryos which slowed growth and conversion into plantlets. Although glucose was less adequate than fructose or sucrose for differentiation at the early stage of asparagus embryos on MM, it was still beneficial in supporting the growth of

roots in mature plantlets and crowns. Other workers have also shown that CHO's may influence morphogenesis per se or through their osmotic properties (Crouch and Sussex, 1981; Kononowicz and Janick, 1984; Levi and Sink, 1990; Strickland et al. 1987; Verma and Dugal, 1977; Wang and Janick,1986).

The role of ABA in reducing callus proliferation at the crown base and increasing root growth might be due to the hormon's effect on cellular osmotic regulation where it inhibits water uptake by the tissue (Schopfer and Plachy, 1984). Still, the role of ABA in normalizing asparagus somatic embryogensis , and in the development and acclimatization of embryo-derived plantlets needs further evaluation.

In the present study, embryogenic suspension cultures were shown to be a potential tool for large scale propagation of mature asparagus crowns. Induction of cultures with NAA at high levels (10-20ppm) enhanced embryo frequency and their capacity to normally convert into plantlets. However, following induction cell lines should be selected prior to production of plantlets.

Embryos formed in suspension had a high conversion rate, where up to 81% of bipolar mature embryos converted into plantlets within ten weeks following their transfer from MM containing 4-10% fructose or sucrose to the 2% medium. This protocol has been successfully used in the propagation of additional elite male lines 88-4 and 88-15 to utilized in our clonal trials.

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APPENDIX

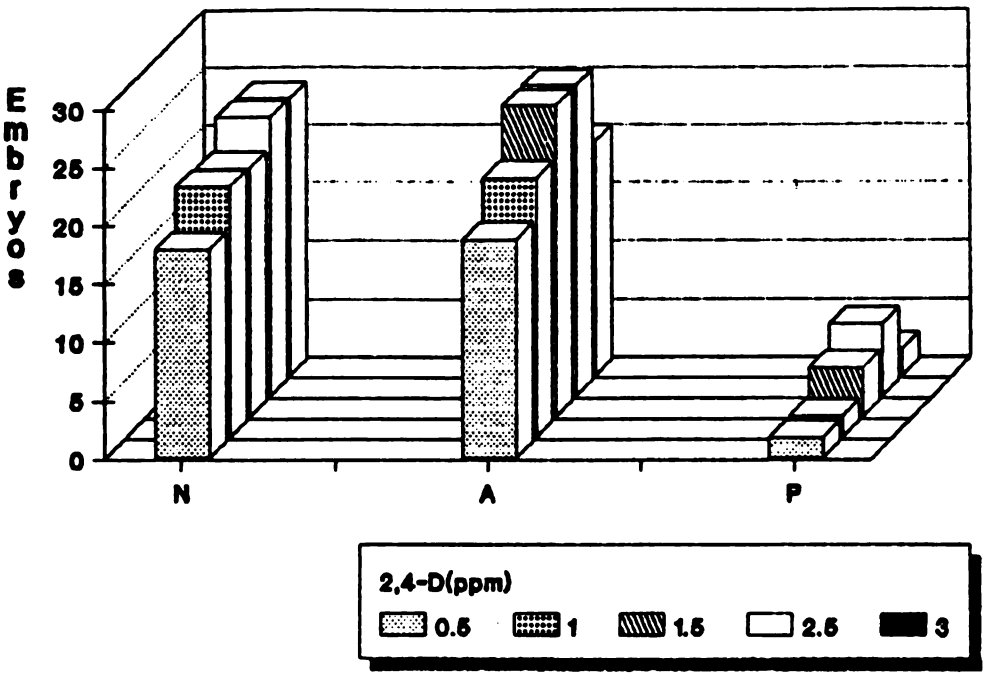

Figure A. Profile of embryos in SS-derived callus induced on MS containing 2,4-D (0.5-3ppm) for four weeks, followed by transfer to NAA (1ppm) for six weeks.

N-normal	A-abnormal	P-plantlets
S.E= 3.1	2.7	0.8

Sorbitol (%)	Sucrose (%)	Shoots	S.D.
4	0	0.25	0.5
3	1	3.75	2.2
2	2	4.60	2.4
1	3	2.35	1.5
0	4	1.30	0.95

Figure B. Combined effect of sorbitol and sucrose combination in MS + (ppm): NAA (0.3) and kinetin (0.8) on formation of shoots in SS-derived callus.

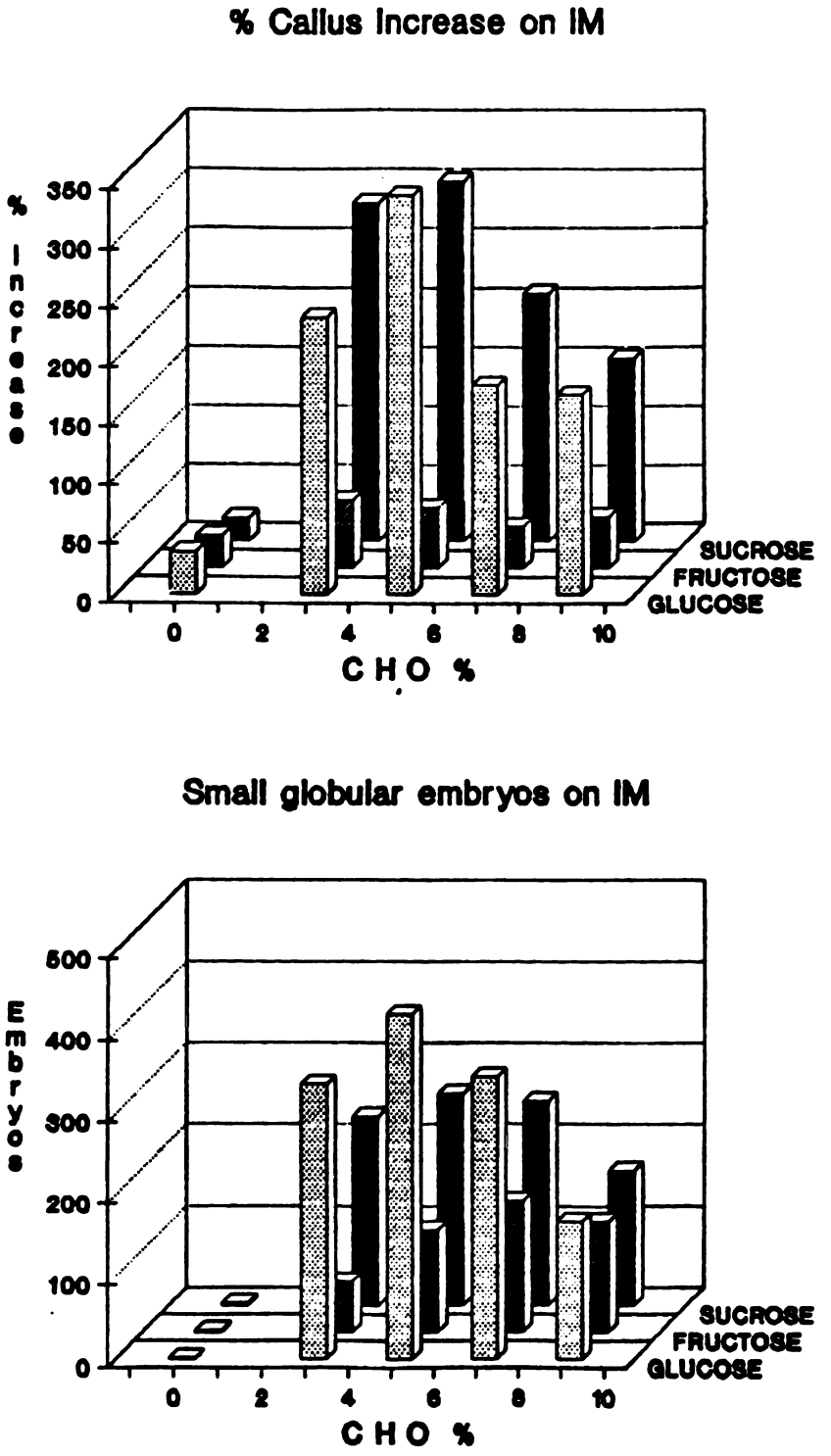

Figure C. Effect of sugar type and concentration in IM containing 2,4-D (1.5ppm) on callus growth and formation of small globular embryoids. No. embryoids per gram callus.

Callus growth: S.E. = 19.3, Embryos: S.E = 10.5; Number of observations = 4.

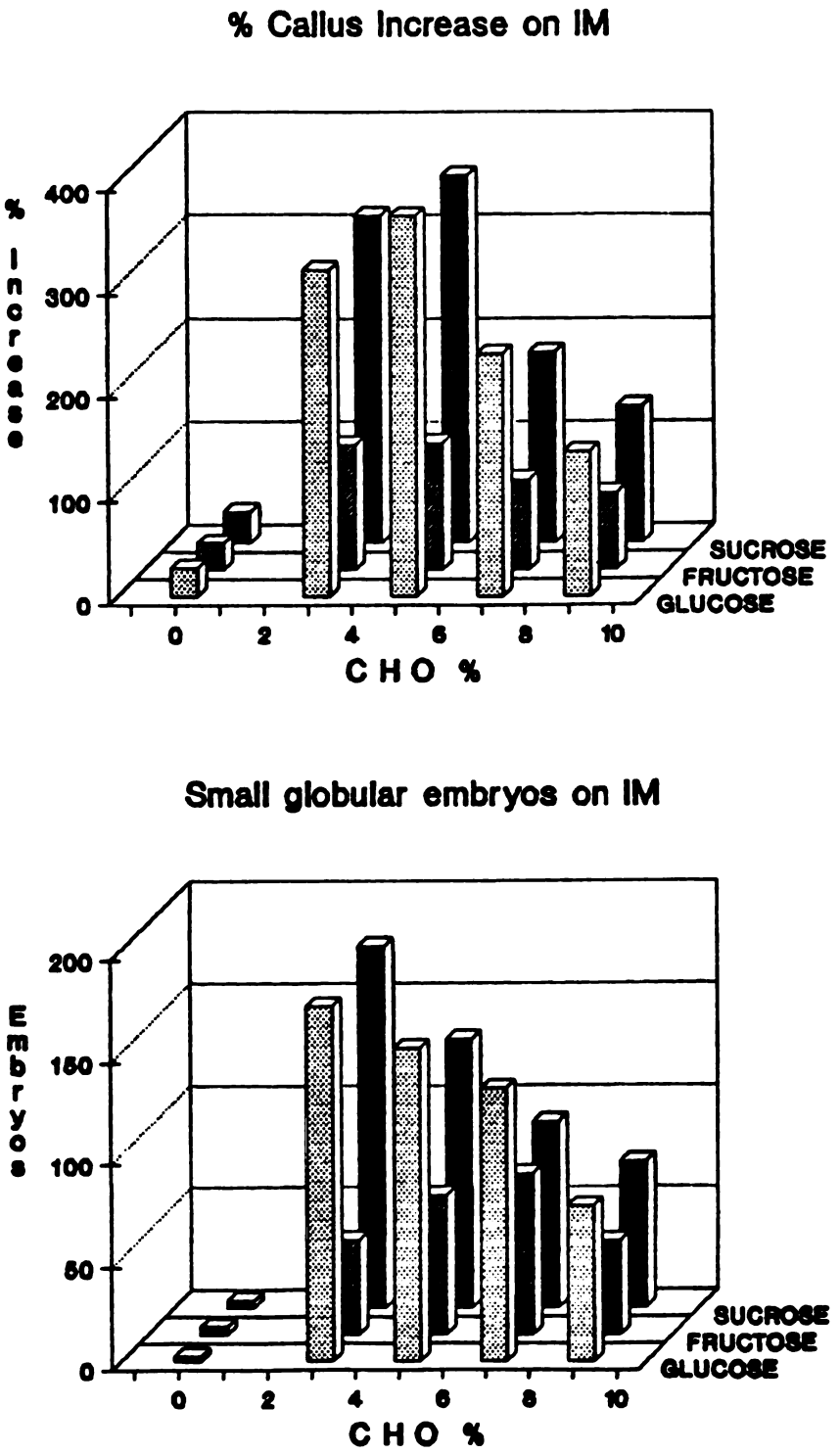

Figure D. Effect of sugar type and concentration in IM containing NAA (1.5ppm) on callus growth and formation of small globular embryoids. No. embryoids per gram callus. Callus growth: S.E. = 23.5, Embryos: S.E = 22.9; Number of observations = 4.