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Investigations into the role of polyamines in strawberry fruit development

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The Ohio State University, 1993
INVESTIGATIONS INTO THE ROLE OF POLYAMINES IN STRAWBERRY FRUIT DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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To My Parents
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INTRODUCTION

Fruit development is a complex process encompassing numerous changes leading to growth, ripening, and senescence. The plant hormones auxin, cytokinin, gibberellin, abscisic acid, and ethylene regulate various aspects of fruit development (Leopold and Kriedemann, 1975). In addition to these hormones, other organic compounds may similarly influence fruit growth and postharvest physiology. Polyamines are naturally occurring compounds that have recently been studied in terms of their potential to affect fruit development (Costa and Bagni, 1983). The most commonly studied of the polyamines are putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM). Since polyamines have been associated with a wide range of physiological processes including cell division, cell expansion, flowering, and response to stress (Galston, 1983), there has been speculation that they may be plant growth substances, similar in function to plant hormones (Bagni, 1989). Endogenous polyamines have been detected in some fruits including apple, avocado, and pear (Biasi et al., 1988; Kushad et al., 1988; Toumadje and Richardson, 1988). There is also evidence that exogenous application of polyamines to fruits can have beneficial effects on growth and postharvest physiology. For example, infiltration of polyamines resulted in increased firmness and longer storage life of fruits such as apple and tomato (Kramer et al., 1991; Law et al., 1991). Whether the observed effects of applied polyamines reflect their normal functions
in fruit development is not known.

There is evidence that polyamines may interact with auxin in regulating plant growth and development. Polyamines were found to substitute for auxin in the initiation of cell division in explants from dormant tubers of *Helianthus tuberosus* (Bagni, 1966; Bertossi et al., 1965). Also, treatment of germinating barley seeds with auxin increased the activity of ornithine decarboxylase, a key polyamine biosynthetic enzyme (Kyriakidis, 1983). In cultured carrot cells, which are induced to differentiate on auxin-containing media, and undergo differentiation into somatic embryos upon transfer to auxin-free media, the levels of certain polyamines increased upon transfer to the medium lacking auxin (Montague et al., 1978). Although these results demonstrate that polyamine levels and enzymes of polyamine metabolism are influenced by auxins, many details pertaining to the interactions between polyamines and plant hormones remain unresolved.

Investigations of polyamine function in fruits, including the possible interactions between polyamines and auxin, may be facilitated by certain characteristics of the strawberry. The strawberry fruit is unique with respect to its morphology and physiology. The fleshy portion of the strawberry is a receptacle, and the true fruits (achenes) are present on the surface of the receptacle. Although in botanical terms the receptacle is not a fruit, it does behave like a true fruit because it undergoes postpollination growth, accumulates sugars, changes color, and ripens. Further, growth of developing strawberry receptacles can be arrested by removal of the auxin-rich achenes from the receptacle surface. Application of auxins to the surface of "de-achedened" receptacles causes re-initiation of growth and results in receptacles similar in
size to normal receptacles (Nitsch, 1950).

The ability to inhibit and re-initiate growth at specific developmental stages, coupled with the relatively short time for completion of fruit development, make the strawberry an attractive system to study auxin-regulated fruit growth. The importance of auxin for development of strawberry receptacles, and previous results implying that polyamines have a hormonal function similar to auxin (Bagni, 1966), led to this study of polyamines in strawberry fruits. In addition, the ability of polyamines to bind to isolated pectin and cell walls (D’Orazi and Bagni, 1987; Scoccianti et al., 1989), and to delay senescence of isolated plant parts (Altman et al., 1977; Davies et al., 1990), prompted investigations into the effects of polyamine treatment on firmness of strawberry fruit tissue. The overall objective of this study was to examine the role of polyamines in strawberry fruit development and postharvest physiology. Specific objectives were:

1. To identify and quantitate polyamines in strawberry receptacles and achenes during development.

2. To investigate the role of polyamines and their interaction with auxin during receptacle development.

3. To determine if polyamines could influence postharvest physiology, specifically tissue firmness, of strawberry.
REVIEW OF LITERATURE

In botanical terms, the fruit is a mature ovary. Esau (1977) provided a broader definition by considering the fruit to be a derivative of the gynoecium and any extracarpellary tissue united with the gynoecium at the fruiting stage. Fruit development usually commences following successful pollination. In some cases, fruit development can also occur without fertilization, as a result of parthenocarpy (Leopold and Kriedemann, 1975). During growth of some fruits such as tomato and apple, a period of cell division is followed by a stage during which enlargement of existing cells occurs (Bohner and Bangerth, 1988; Leopold and Kriedemann, 1975). On the other hand, cell division is thought to occur in avocado as long as the fruit is attached to the plant (Kushad et al., 1988). Regardless of the pattern of cell division, ripening usually occurs towards the end of the growth period, and is characterized by fruit softening, sugar accumulation, and changes in color and flavor.

Fruit growth and postharvest physiology are influenced by plant hormones. For example, endogenous auxins, gibberellins, and cytokinins influence fruit set and growth, and exogenous application of these compounds can also significantly affect development (Leopold and Kriedemann, 1975; Mudge et al., 1981). In terms of postharvest physiology, ethylene is an important regulator of fruit ripening (Knee, 1989). A relatively recent development has been the interest in polyamines as natural regulators
of fruit growth and ripening (Costa and Bagni, 1983; Saftner and Baldi, 1990). The pertinent literature regarding polyamines in fruit development is summarized below, along with information concerning development of strawberry fruits.

Polyamines and their metabolism in plants

The polyamines PUT, SPD, and SPM are commonly found in plants and have been associated with a wide range of developmental processes. CAD is less common, but has also been found in some plants. Early studies by Bagni and co-workers on growth of *Helianthus tuberosus* tubers suggested a role for polyamines in plant development (Bagni, 1966; Bertossi et al., 1966). Subsequently, Galston's group while investigating viability of cereal protoplasts, found that polyamines were capable of significantly delaying protoplast senescence (Altman et al., 1977). These results led to numerous investigations into the possible growth regulatory properties of polyamines in plants (reviewed by Galston, 1983; Evans and Malmberg, 1989).

The biosynthesis and catabolism of polyamines in plants has been reviewed elsewhere (Smith, 1985), and is briefly summarized here. PUT is synthesized via ornithine decarboxylase or arginine decarboxylase (Fig. 1). Fungi and animals have ornithine decarboxylase, but lack arginine decarboxylase (Tabor and Tabor, 1984). The reason for the two pathways for PUT biosynthesis in plants is not completely understood. Although exceptions have been reported (Minocha et al., 1991), ornithine decarboxylase may be responsible for PUT synthesis during cell division, whereas arginine decarboxylase has been associated with stress responses (Evans and Malmberg, 1989).
Figure 1. Pathways for biosynthesis of polyamines in plants. Key enzymes are 1, ornithine decarboxylase; 2, arginine decarboxylase; 3, S-adenosylmethionine (SAM) decarboxylase; 4, spermidine synthase; 5, spermine synthase; 6, lysine decarboxylase. Not all intermediates have been represented in the figure.
PUT is converted to SPD by SPD synthase following the addition of a propylamino group from decarboxylated S-adenosylmethionine. Similarly, SPM synthase is responsible for the addition of a propylamino group to SPD. Since S-adenosylmethionine (SAM) is a common intermediate for the biosynthesis of polyamines and ethylene, the possibility that metabolic competition exists for this intermediate has been raised by several researchers (Apelbaum et al., 1981; Winer and Apelbaum, 1986). CAD is formed in a separate pathway from lysine in a reaction catalyzed by lysine decarboxylase. Polyamines are catabolized by diamine or polyamine oxidases, which have been mostly studied in legumes and cereals (reviewed by Smith, 1985; Suzuki et al., 1990).

**Polyamines in fruit set and fruit development**

Exogenous application of polyamines to fruits resulted in beneficial effects on fruit set and yield in some studies. Costa and Bagni (1983) reported that sprays of aqueous solutions of PUT, SPD, and SPM at relatively low concentrations (1 or 10 μM) shortly after full bloom resulted in improved fruit set and yield of apples. Biasi et al. (1991) also reported increases in fruit set, yield, and weight of apples following sprays of PUT at full bloom. However, increased fruit set and yield have not always been observed as a result of polyamine treatment. For instance, Miller et al. (1988) found that sprays of polyamines had no significant effect on fruit set, yield, shape, or weight of apple fruits in a multiple year trial. Volz and Knight (1986) observed a similar lack of effect when PUT was sprayed on trees of several apple cultivars. In olive, PUT sprays increased fruit set and yield (Rugini and Mencuccini, 1985; Rugini et al., 1986). Although fruit
set in olive was stimulated by high concentrations of PUT (50 mM), PUT caused a
decrease in weight of olive fruits (Rugini and Mencuccini, 1985). PUT sprays also
increased fruit set of 'Comice' pears without affecting fruit weight (Crisosto et al.,
1988). In this study, PUT (1 mM) extended ovule longevity, but did not reduce ethylene
evolution from pollinated flowers.

Endogenous (free) polyamines levels have been measured during growth of some
fruits including apple, avocado, citrus, and pear (Biasi et al., 1991; Kushad et al., 1988;
Nathan et al., 1984; Toumadje and Richardson, 1988). In general, PUT, SPD, and SPM
are the common polyamines that have been detected in most fruits. CAD is less
common, but has been reported in 'Rutgers' tomato fruits (Bakanashvili et al., 1987;
Saftner and Baldi, 1990). Polyamine concentrations have usually been expressed on a
nmol/g fresh weight basis in many of these studies, and their concentrations are usually
higher during the early stages of fruit growth, and decline to lower levels later in
development. Individual polyamine concentrations during the early stages of
development often range from 200 to 500 nmol/g fresh weight, and are usually
considerably below 100 nmol/g fresh weight at harvest. Differences in the polyamine
profile within cultivars of a species have been reported. For example, levels of SPM and
PUT were similar (>200 nmol/g fresh weight) in 'Comice' pears early in development,
but SPM was undetectable in 'Bartlett' fruits at a comparable developmental stage
(Toumadje and Richardson, 1988). Concentrations of PUT, SPD, and SPM increased
in avocado (cultivar Simmonds) shortly after full bloom, then continuously declined
during development (Kushad et al., 1988). In contrast, Winer and Apelbaum (1986)
found that although PUT and SPD concentrations declined during the course of avocado (cultivar Fuerte) development, SPM levels showed no change.

In some cases, polyamine conjugates have been studied in addition to free (soluble) polyamines. The significance of these conjugates is not known, although they have been considered to be storage forms for the free polyamines (Slocum and Galston, 1985a). In members of the Solanaceae, such as tobacco and tomato, substantial amounts of acid-soluble and acid-insoluble conjugates are present in fruits (Egea-Cortines et al., 1990; Slocum and Galston, 1985a). However, in 'Golden Delicious' apples, although acid-insoluble conjugates were present and exhibited similar developmental changes as the free polyamines, acid-soluble conjugates were not detected (Biasi et al., 1988). In a subsequent investigation with 'Topred' apples, Biasi et al. (1991) studied only the free polyamines, which they considered to be the metabolically active form.

Arginine decarboxylase and ornithine decarboxylase have been the most commonly studied polyamine biosynthetic enzymes in fruit development (Winer et al., 1984; Winer and Apelbaum, 1986; Kushad et al., 1988). Tomato ovaries exhibited a rapid elevation in ornithine decarboxylase activity shortly after pollination or treatment with auxin (Heimer et al., 1979; Mizrahi and Heimer, 1982). In tobacco, activity of ornithine decarboxylase is greater than that of arginine decarboxylase throughout ovary development (Slocum and Galston, 1985a). Although ornithine decarboxylase appears to be the major enzyme responsible for PUT synthesis during the early stages of tomato and tobacco development, this trend may not be consistent with fruits of other species. For example, higher arginine decarboxylase activity was found in comparison with that of
ornithine decarboxylase in apple and citrus fruits (Biasi et al., 1991; Nathan et al., 1984). Thus, the relative importance of the two pathways for PUT biosynthesis in fruits remains unclear, and seems to vary with species. The potential interference due to non-enzymatic decarboxylation of labelled amino acids during the conventional 14CO2-trapping enzyme assays could affect measurement of decarboxylase activity in plants (Smith and Marshall, 1988a,b), and should be taken into consideration when measuring activities of these enzymes in fruits.

Attempts have been made to study a role for polyamines in fruits by inhibition of polyamine biosynthesis. Inhibition of tomato fruit growth during the early stages of development was accomplished by feeding inhibitors of ornithine decarboxylase, difluoromethylornithine (DFMO) or α-methylornithine, via the inflorescence stalk (Cohen et al., 1982). These inhibitors reduced the activity of ornithine decarboxylase in fruit tissue, although their effect on PUT levels was not presented. The effects of these inhibitors on growth could be reversed by simultaneously supplying PUT with the inhibitors. Teitel et al. (1985) determined that treatment with DFMO reduced tomato fruit weight and the number of cells in fruits, and these effects could be reversed by simultaneous treatment with PUT. In a more recent study, Egea-Cortines et al. (1990) reported that DFMO was more effective at inhibiting growth of pollinated, rather than auxin-induced (parthenocarpic) fruits. Therefore, DFMO may have affected embryogenesis in pollinated fruits, thereby leading to greater inhibition of growth in pollinated, but not auxin-induced (seedless) fruits. Polyamine biosynthetic inhibitors also reduced growth of tobacco ovaries (Slocum and Galston, 1985b). However,
simultaneous supply of PUT with the inhibitors did not completely reverse the effects of the inhibitors on tobacco ovary growth. Growth of apple fruits was not inhibited following multiple dips in solutions of polyamine biosynthetic inhibitors (Miller et al., 1988).

**Polyamines in postharvest physiology**

Polyamines have been studied in relation to postharvest physiology in some fruits. Many of these studies have examined the possible involvement of polyamines in the delay of senescence, maintenance of firmness, and reduction of chilling injury.

Since ethylene and polyamines apparently have antagonistic effects on plants, and share common biosynthetic intermediates, some postharvest research in fruits has centered around possible interactions between ethylene and polyamines. Exogenous application of polyamines to isolated plant parts appears to have an inhibitory effect on ethylene biosynthesis. For example, polyamines inhibited biosynthesis of ethylene in apple fruit tissue and protoplasts (Apelbaum et al., 1981). Similarly, incubation of avocado fruit discs with polyamines reduced ethylene production (Winer and Apelbaum, 1986). Biosynthesis of ethylene was inhibited and incorporation of 3,4-[14C]methionine into SPD increased, upon treatment of aged orange peel discs with PUT or SPD (Even-Chen et al., 1982). Li et al. (1992) recently found that incubation of tomato slices with polyamines reduced 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, and accumulation of a wound-induced ACC synthase transcript, again demonstrating a possible link between polyamines and ethylene in fruits. Inhibition of ethylene
biosynthesis by spermidine was also reported in suspension-cultured cells of pear fruits (Ke and Romani, 1988).

Although the studies described above indicate that polyamines are capable of reducing ethylene biosynthesis in isolated fruit tissue, it remains unclear as to whether polyamines regulate ethylene biosynthesis in vivo. Some studies indicate that higher levels of polyamines are present at times of reduced ethylene synthesis. In certain tomato cultivars, reduced rates of ripening were correlated with elevated levels of polyamines. Saftner and Baldi (1990) found that the slow-ripening tomato cultivar, Liberty, which produced less ethylene from pericarp slices, contained higher levels of polyamines than more rapidly ripening cultivars. Tomato fruits of the landrace Alcobaca, which had prolonged keeping qualities, also contained higher levels of PUT in ripe fruits, in comparison with a normal ripening mutant of Alcobaca (Alcobaca-red) (Dibble et al., 1988). The high levels of PUT in slow-ripening tomatoes was attributed to increased activity of arginine decarboxylase, and not to reduced catabolism of PUT (Rastogi and Davies, 1991). In pears which were unable to ripen or produce ethylene because of unfulfilled chilling requirements, SPM levels remained high. In contrast, SPM concentrations in ethylene-producing pears declined significantly during ripening, leading to speculation that SPM may regulate ethylene metabolism during ripening of pears (Toumadje and Richardson, 1988).

Unlike the results discussed above, which imply a relationship between polyamines and ethylene, some studies suggest that polyamines and ethylene do not compete for the same substrates during fruit ripening. The levels of PUT and SPD in
'Lorena' tomatoes were not significantly altered during establishment of the climacteric peak of ethylene (Casas et al., 1990). The authors concluded that accumulation of ACC in these tomato fruits was not a consequence of decreased SPD synthesis. Similarly, from a study of polyamine and ethylene biosynthesis in avocado, Kushad et al. (1988) concluded that polyamines and ethylene did not compete for the same substrates during fruit ripening. Comparisons between normal apple fruits and fruits affected with watercore revealed that watercore-affected fruits contained higher levels of polyamines and ethylene than normal fruit (Wang and Faust, 1992). Both normal and watercore-affected fruits contained similar levels of SAM in this study, and SAM levels did not vary at times of active ethylene synthesis and polyamine accumulation.

Polyamines have also been studied in relation to chilling injury of fruits. Fruits of lemon, grapefruit, and pepper that were subjected to conditions that resulted in chilling injury, accumulated greater amounts of PUT than fruits stored at higher temperatures (McDonald and Kushad, 1986). In this study, PUT concentrations in lemon flavedo were highly correlated with the severity of chilling injury, leading to the suggestion that PUT could be the cause of chilling injury. Exposure of zucchini squash to chilling temperatures also resulted in the accumulation of PUT and SPD (Wang and Ji, 1989). However, chilling injury was reduced, and levels of SPD and SPM were higher in low-oxygen stored fruits, when compared to fruits stored in air. Temperature preconditioning of zucchini squash prior to low temperature storage also resulted in increased synthesis of SPD and SPM, and a reduction in chilling injury (Kramer and Wang, 1989; Kramer and Wang, 1990). Therefore, the authors concluded that SPD and SPM may have a
protective function in reducing chilling injury in zucchini squash.

Further evidence that polyamines may be involved in postharvest physiology was presented by Kramer et al. (1989), who found reduced rates of softening and elevated polyamine levels in apple fruits subjected to low-oxygen storage. Polyamines also reduced the in vitro activity of polygalacturonase extracted from apples that had been inoculated with *Penicillium*, leading to speculation that polyamines protect cell walls from wall-degrading enzymes (Kramer et al., 1989).

Exogenous application of polyamines may have beneficial effects on the storage life of fruits. Kramer et al. (1991) demonstrated that pressure infiltration with polyamines caused an immediate increase in apple fruit firmness, and reduced the rate of softening during postharvest storage at low temperature. In this study, the authors concluded that polyamines may have delayed softening by affecting the rigidity of cell walls of treated fruit. Vacuum infiltration of polyamines into tomatoes also increased the storage life of treated fruits (Davies et al., 1990; Law et al., 1991). The polyamine treatments significantly retarded fruit softening in these studies. Exogenous application of SPM to zucchini squash also had a beneficial effect by causing a reduction in the incidence of chilling injury under low temperature storage (Kramer and Wang, 1989).

**Strawberry fruit development**

The cultivated strawberry (*Fragaria x ananassa* Duch.) is a dicotyledonous angiosperm belonging to the family Rosaceae. Strawberry cultivars are derived almost exclusively from *F. chiloensis* and *F. virginiana* (Galletta and Bringhurst, 1990).
Strawberry plants are herbaceous perennials and the strawberry inflorescence is classified as a compound dichasium (Galletta and Bringhurst, 1990). Although some strawberry species are dioecious, many cultivars bear complete and perfect flowers. Several hundred pistils are inserted into the surface of the swollen tip of the pedicel (receptacle), with the ovaries connected to the receptacle by a vascular trace. Surrounding the receptacle are arranged the stamens, in multiples of five, often numbering 25-30. The corolla, usually consisting of five white petals is present outside the stamens. The calyx, consisting of one or two rings with five sepals each, is located outside the petals (Galletta and Bringhurst, 1990).

The strawberry ‘fruit’ is an aggregate fruit that consists of the achenes (or true fruits), which are present on the surface of the receptacle (Esau, 1977). Although the receptacle is not a true fruit from a botanical viewpoint, following pollination it grows, accumulates sugars and ripens very much like a true fruit (Knee et al., 1977). The stage of fruit development is often classified based on color as green, white, or red. Chlorophyll concentration is high during the green stage, and progressively declines during the white and red stages, whereas anthocyanin concentration increases dramatically at the red stage (Cheng and Breen, 1991). Fruits are usually ripe 25 d after pollination, although the ripening period can vary depending on cultivar and environmental condition (Galletta and Bringhurst, 1990).
Hormonal control of strawberry fruit development

Nitsch (1950) found that removal of the achenes from the surface of developing receptacles, caused inhibition of receptacle growth. Application of the synthetic auxin β-naphthoxyacetic acid to the surface of the de-achened receptacles caused re-initiation of growth leading to the hypothesis that the achenes were supplying auxin to the developing receptacle. In support of this hypothesis, bioassays revealed that receptacles lacked free auxin activity, whereas achenes proved to be rich source of free auxin (Nitsch, 1950; Nitsch, 1955). Subsequent studies provided mass spectral confirmation for the presence of indole acetic acid (IAA) in strawberry fruits (Dreher and Poovaiah, 1982). IAA was found at the highest concentration 10 d after pollination in the achenes, but was present in only trace amounts in receptacles at the same stage of development (Dreher and Poovaiah, 1982). Archbold and Dennis (1984), using gas chromatography-mass spectrometry reported considerably higher concentrations of free IAA in achenes than in receptacles 4-20 d after anthesis.

In contrast to reports indicating absence or only trace amounts of free IAA in receptacle tissue 10-12 d after pollination (Nitsch, 1950; Nitsch, 1955; Dreher and Poovaiah, 1982), similar concentrations of IAA were reported 12 d after pollination in both achene and receptacle tissue by Archbold and Dennis (1985). However, removal of achenes from the receptacle surface inhibited growth and caused auxin concentrations in the receptacle to dramatically decrease to low or undetectable levels two days after achene removal (Archbold and Dennis, 1985).

In addition to their importance for receptacle growth following removal of
achenes, auxins are capable of inducing fruit development in the absence of pollination. Treatment of unpollinated flowers with synthetic auxins resulted in the development of parthenocarpic fruits that were similar in size or smaller than pollinated fruits (Archbold and Dennis, 1985; Mudge et al., 1981; Thompson, 1967).

The ability to arrest growth by removal of achenes at various stages of development, along with the capacity to re-initiate receptacle growth by supplying auxins has led to the use of the strawberry as a model system for the investigation of hormonal regulation of fruit growth (Given et al., 1988; Jena et al., 1989; Lis and Antoszewski, 1982). Of a number of auxins and structurally related compounds tested, α-NAA was the most effective at inducing growth of de-achened strawberry receptacles, whereas structurally similar non-auxins were ineffective (Mudge et al., 1981). The ability of auxins to bind to sites in receptacle membrane fractions was positively correlated with growth promoting activity of the compounds tested, implying that these auxin-binding sites could have physiological significance as receptors (Narayan et al., 1981). In addition to affecting the early stages of fruit growth, auxins may also control ripening during later stages of development. The non-climacteric nature of the strawberry fruit, and the ability of auxin to inhibit anthocyanin biosynthesis and softening in de-achened receptacles, has given rise to the concept that auxin rather than ethylene may regulate the rate of ripening (Given et al., 1988).

Although the studies described above demonstrate the importance of auxins for growth of strawberry receptacles, the possibility that other growth regulators also have a role in fruit development cannot be overlooked. Lis et al. (1978) used bioassays to
demonstrate the presence of cytokinin, gibberellin, and abscisic (ABA) acid-like compounds in achenes and receptacles. The presence of free ABA in strawberry fruits was confirmed by gas chromatography (Archbold and Dennis, 1984). In general, exogenous auxin has a greater effect on receptacle growth and development than other hormones. For example, gibberellins (GA$_{4+7}$ or GA$_3$) were ineffective at promoting growth of de-achened receptacles (Archbold and Dennis, 1985). Tafazoli and Vince-Prue (1979) reported that GA$_3$ was less effective than IAA at stimulating growth of receptacles de-achened 9 d after pollination. Similarly, GA$_3$ or kinetin did not cause the accumulation of radioactively labelled assimilates, whereas IAA was highly effective at inducing the accumulation of labelled assimilates in receptacle tissue devoid of achenes (Lis and Antoszewski, 1979). Gibberellins are also less effective than auxins in terms of their ability to promote parthenocarpic fruit development. Treatment of the flowers of the pistillate strawberry cultivar Freya with auxin resulted in fruits similar in size to pollinated controls, whereas gibberellin-induced growth was limited to the 'neck' region below the point of attachment of the carpels (Thompson, 1967). In addition, GA$_3$ or GA$_{4+7}$ were less effective than auxin at stimulating parthenocarpic fruit development in emasculated flowers (Archbold and Dennis, 1985), thereby confirming the importance of auxin for fruit development.

Ripening and postharvest physiology of strawberry

The strawberry fruit is non-climacteric and exhibits a continuous decline in ethylene production during the course of development (Knee et al., 1977). Softening of
the ripe fruit does not appear to be a result of the activity of polygalacturonase (Barnes and Patchett, 1976; Huber, 1984; Neal, 1965). Neal (1965) concluded that cell separation during ripening may be due to a change in the cationic content of the middle lamella rather than due to degradation of pectin molecules. Ripe strawberry tissue softened upon treatment with ethylenediaminetetraacetic acid (EDTA), and was re-firmed following treatment with salts of divalent cations (Neal, 1965). A number of studies have since demonstrated the firming effect of calcium salts on strawberry tissue. Rosen and Kader (1989) found no differences in firmness of strawberry slices immediately following dip-treatment in water or calcium chloride solution. However, after storage at 2.5 °C for 7 d, calcium-treated slices were firmer than the slices dipped in water or whole, freshly sliced fruit. Treating strawberries with calcium lactate solutions also improved firmness and character, presumably as a result of binding of calcium to pectins in the middle lamella (Main et al., 1986; Morris et al., 1985). Foliar application of calcium reduced softening of strawberries stored at 4 °C (Cheour et al., 1991). In this study, calcium was found to have a greater effect on fruit of ‘Glooscap’, a relatively soft- fruited cultivar, in comparison with fruit of ‘Kent’, a cultivar with firmer fruit. Many studies of polyamines and their effects on firmness and shelf-life have been carried out with climacteric fruits such as apples and tomatoes (Davies et al., 1990; Kramer et al., 1991; Saftner and Baldi, 1990). However, the importance of cations for maintenance of fruit firmness (Neal, 1965), suggest that polyamines could also influence firmness of non-climacteric fruits such as strawberries.
CHAPTER I

DEVELOPMENTAL AND AUXIN-INDUCED CHANGES IN POLYAMINE LEVELS DURING STRAWBERRY FRUIT DEVELOPMENT

Abstract

Polyamines have been associated with a wide array of physiological processes in plants, thereby leading to speculation that they have a role similar to that of plant hormones, or that they may mediate the effects of plant hormones. The possible involvement of polyamines during strawberry (*Fragaria x ananassa* Duch.) fruit development, a process strongly influenced by auxin, was investigated in this study. Putrescine, spermidine, and spermine were identified in strawberry receptacles and achenes at all stages of development. Total (free) polyamine concentrations decreased from a maximum of 485 nmol/g fresh weight at pollination to a minimum of 55 nmol/g fresh weight in ripe receptacles. Total polyamine concentrations during corresponding stages of development were consistently higher in achenes than in receptacles, and ranged from 891 to 203 nmol/g fresh weight. Removal of achenes from the surface of developing receptacles 10 d after pollination reduced receptacle growth, and re-initiation of growth by application of auxins was accompanied by a rapid, approximately 1.5-fold elevation in polyamine concentrations 24 h after treatment. The auxin, $\alpha$-naphthaleneacetic acid ($\alpha$-NAA), increased growth and polyamine levels to a greater
extent than the structurally related, but less effective auxin, β-NAA. Polyamine concentrations in receptacles with intact achenes remained similar to those of auxin-depleted (de-achedened) receptacles, implying that these compounds may not be limiting during the initial stages following achene removal. In addition, since application of polyamines to de-achedened receptacles did not initiate growth, polyamines may not play a hormonal role such as auxin under these conditions. Polyamine content per receptacle increased >3-fold in normally developing receptacles and auxin-treated receptacles 10 d after removal of achenes, but did not increase during this period in auxin-depleted receptacles. Although polyamines may not have a hormonal role in this system, their levels are influenced by auxin and they may be important for continued receptacle development.
INTRODUCTION

Polyamines are thought to influence various aspects of plant development such as cell division, growth, morphogenesis, and response to stress (Galston, 1983). The range of processes that these poly-cations have been associated with has led to the idea that they may be hormonal second messengers (Galston, 1983), or even a separate class of plant growth substances (Bagni, 1989).

Recent research has suggested a role for polyamines in both fruit growth and postharvest physiology. In a number of fruits such as apple (Biasi et al., 1988), avocado (Kushad et al., 1988), and pear (Toumadje and Richardson, 1988), polyamine concentrations on a fresh weight basis were highest at early stages of development and thereafter declined to lower levels. However, a clear role for polyamines in the development of these fruits has not been defined. Polyamine metabolism in fruits has probably been investigated to the greatest extent in tomato. Treatment of unpollinated tomato flowers with synthetic auxins induces parthenocarpic fruit development. Such auxin-induced fruits exhibited higher ornithine decarboxylase activity when compared with unpollinated control fruits (Mizrahi and Heimer, 1982). In addition, application of inhibitors of ornithine decarboxylase activity to pollinated tomato fruits inhibited growth, and this inhibition was reversed by the simultaneous application of putrescine, thus suggesting a role for ornithine decarboxylase early in tomato fruit development (Cohen et al., 1982). However, in a more recent study Egea-Cortines et al. (1990) found that difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine
decarboxylase, had a much lower inhibitory effect on growth of auxin-induced fruits (27% inhibition) in comparison with its effect on normally pollinated fruits (73% inhibition). These results raise the possibility that the main effect of DFMO on tomato fruits may be due to inhibition of seed development, which in turn results in reduced fruit growth.

Polyamines may also influence the postharvest physiology of fruits. In tomato, certain cultivars that had improved keeping quality also had higher levels of endogenous polyamines (Dibble et al., 1988). Application of polyamines to fruits also affects their physiology during storage. For example, infiltration of polyamine solutions resulted in increased storage life of tomatoes (Law et al., 1991). Similarly, infiltration of polyamines also caused an increase in firmness of apple fruits (Kramer et al., 1991).

The strawberry fruit, because of its morphology and physiology, presents a unique system to study fruit development. The fleshy portion of the strawberry is a receptacle and the true fruits (achenes) are present on the surface of the receptacle. However, the receptacle does behave like a fruit because following pollination it grows, accumulates sugars, changes colour, and softens. The achenes are a rich source of auxin and removal of achenes from the surface of the enlarging receptacle causes inhibition of growth that can be reversed by the application of auxin to the surface of the "de-acheden" receptacle (Nitsch, 1950).

The ability to arrest growth at various developmental stages and to re-initiate growth of de-achedened receptacles, make the strawberry an attractive system to study auxin-induced fruit growth. In addition, the relatively short time for completion of
postpollination fruit development (25-30 d), and previous results linking polyamines with auxin (Bagni, 1966; Mizrahi and Heimer, 1982; Kyriakidis, 1983), led us to investigate polyamine metabolism in strawberry fruits. The initial objective of this study was to identify the polyamines and determine changes in their levels in relation to strawberry fruit development. Because of the importance of auxin for receptacle growth, the possibility that polyamines are subject to regulation by auxins during receptacle development was also investigated.

MATERIALS AND METHODS

Chemicals

Polyamine standards (hydrochloride salts), α-naphthaleneacetic acid (α-NAA), β-naphthaleneacetic acid (β-NAA), indole-3-acetic acid (IAA), dimethylsulfoxide (DMSO), mannitol and Na₂-EDTA were purchased from Sigma Chemical Company, St. Louis, MO. Lanolin (anhydrous) was obtained from Mallinckrodt, Paris, KY. Pectinase was obtained from Nutritional Biochemicals Corp., Cleveland, OH. Difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA) were gifts from Merrell-Dow Research Institute, Cincinnati, OH.

Plant material

Strawberry (Fragaria x ananassa Duch., cultivar Allstar) plants were obtained from Brittingham Plant Farms, Salisbury, MD. Plants were grown in 15 cm-tall
(approximately 1.5 L) pots containing (by volume) sand (33%), silt loam (33%), vermiculite (11%), peat moss (11%) and perlite (11%). The plants were grown under a 16 h light/8 h dark photoperiod in a greenhouse. Liquid fertilizer (20-20-20; N-P-K) was supplied on a weekly basis. In addition, each pot was treated with a single application (3 g) of Osmocote 19-6-12 (N-P-K) fertilizer (Sierra Chemical Company, Milpitas, CA) one week after planting. Fully opened primary flowers were pollinated with a paint brush and tagged. In some studies, achenes from primary fruits at the green-stage of development were gently removed with a blunt spatula and lanolin pastes were applied to the surface of the de-achened receptacles. Treatment and harvest of fruits was carried out towards the end of the light period. All flowers or fruits other than the primary fruit were removed from the inflorescence. For experiments in which fruit/receptacle diameter was reported, the maximum diameter of the fruit/receptacle was measured to the nearest 0.5 mm with vernier calipers. Similarly, fruit/receptacle length was measured as the distance between the apex and the point of attachment of the calyx to the pedicel.

Determination of cell number in receptacles

Fruits at the appropriate stage of development were harvested and receptacles were separated from other floral parts. Receptacles were sliced into smaller sections and the tissue was macerated by mixing with a solution (0.1 ml solution/mg tissue) of 0.1 M Na₂-EDTA, 0.4 M mannitol, and 3.5% (w/v) pectinase, at pH 4 (Bohner and Bangerth, 1988). The mixture was shaken in a water bath overnight at 32 °C, and larger clumps
of undigested tissue were gently disrupted with the help of a syringe fitted with an 18-gauge needle. The suspension was then diluted with water or concentrated by centrifugation, and cells were counted in a Sedgewick-Rafter counting chamber (50 mm x 20 mm x 1 mm) according to Dodds and Roberts (1985).

**Lanolin pastes**

In experiments where only auxins were applied as lanolin pastes, the auxins (IAA/NAA) were dissolved in DMSO, and an aliquot of the solution was thoroughly mixed with molten (50 °C) lanolin, so that the final concentration of auxin was 1 mM. In all experiments, the final concentration of DMSO in the lanolin pastes was 2%; therefore 'control' receptacles were de-achedened and treated with lanolin containing 2% DMSO.

**Polyamine analysis**

Tissue was frozen in liquid nitrogen and stored at -20 °C. If receptacles had been treated with lanolin, the lanolin was removed from the surface by wiping with tissue paper prior to freezing. Soluble polyamines were extracted by homogenizing tissue in ice-cold (5%) perchloric acid (100-200 mg fresh weight tissue/ml perchloric acid). Prior to homogenization, 1,8-diaminooctane was added as an internal standard. The homogenate was incubated at 4 °C for 1 h, then centrifuged at 20,000 xg for 25 min at 4 °C. The volume of supernatant was measured prior to storage in plastic vials at -20 °C. Free polyamines in the supernatant were determined by HPLC following
benzoylation. Briefly, 250-500 µl of the perchloric acid extract was mixed with 1 ml 2N NaOH in a 19 x 45 mm screw-cap vial. Benzoyl chloride (10 µl) was added and the contents were vortexed for 10 s. The mixture was incubated for 20 min at room temperature prior to addition of 2 ml saturated NaCl. Diethyl ether (2 ml) was then added to the mixture, vials were sealed with teflon lined caps, vortexed for 10 s, and centrifuged (1500 xg) for 5 min. One ml of the ether phase was removed and dried under a stream of air. The residue was redissolved in methanol and benzoyl polyamines separated by HPLC (Slocum et al., 1989; gradient elution program I) using a 250 x 4.5 mm octadecyl column (Nicolet Instrument Corp., Madison, WI) and Ternary Gradient Liquid Chromatograph (IBM Instruments; LC/9533) equipped with a 254 nm IBM UV detector (LC/9522).

RESULTS

Developmental study. Following pollination, receptacle development proceeded rapidly with the change in fresh weight being characterized by a sigmoidal growth curve (Fig. 2A). Fresh weight increased from 17 mg on the day of pollination to a maximum of 8.72 g on day 25 when receptacles were at the red-ripe stage of development. Receptacles exhibited visible signs of senescence 30 d after pollination. Changes in cell number of 'Allstar' receptacles were determined in order to establish the cell division phase of development. The number of cells per receptacle increased until 10 d after pollination and remained fairly constant during subsequent development. Cell number
Fig. 2A,B. Postpollination development of strawberry receptacles. A. Changes in receptacle fresh weight (■) and diameter(▼); each point represents the mean value for n ≥ 9 receptacles. B. Changes in cell number/g fresh weight (+) and cell number per receptacle (♦); each point represents the mean for n ≥ 3 replicate samples, each replicate consisting of 1 or more receptacles; vertical bars indicate the SE.
expressed on a fresh weight basis, continued to decline during receptacle enlargement from a maximum value on the day of pollination to a minimum in ripe fruits (Fig. 2B). These results, which suggest that the majority of cell division in receptacles is completed about 10 d following pollination are in agreement with those of Knee et al. (1977) and Cheng and Breen (1992). Cell numbers per ripe strawberry receptacle of ‘Allstar’ (1.45 x 10⁶) are similar to those reported for ‘Tristar’ (1.96 x 10⁶) by Cheng and Breen (1992) but are less than those reported for ‘Prizewinner’ (3.32 x 10⁶) by Knee et al. (1977). As pointed out by Cheng and Breen (1992), such variations in cell number in fruits of similar weight may be a consequence of differences in growth conditions, method of maceration, or genotype.

The polyamines, putrescine (PUT), spermidine (SPD), and spermine (SPM) were present at all stages of achene and receptacle development, but cadaverine was not detected. Since many of the positive effects of polyamines on growth have resulted from supply of these compounds in free form (Bagni, 1966; Jarvis et al., 1983), this study of polyamines in strawberry fruits was concerned with free, but not conjugated/bound forms of these compounds. It is conceivable however, that conjugated/bound forms of polyamines serve important functions in development, although this possibility was not investigated in strawberry fruits. On a fresh weight basis, mean total polyamine (PUT + SPD + SPM) concentrations were highest at the early stages of receptacle development (485 nmol/g on day of pollination) and declined to a minimum (55 nmol/g) in ripe fruits (Fig. 3A). Levels of PUT declined more gradually and remained higher than those of SPD and SPM between 5 and 15 d after pollination; however, SPD was the
Fig. 3A,B. Changes in free polyamine (PA) concentrations in strawberry (A) receptacles and (B) achenes during development. Mean levels of PUT (△), SPD (○), and SPM (▲) for n ≥ 3 replicate samples are represented by each point; vertical bars indicate the SE.
predominant polyamine in ripe receptacles. Total (mean) polyamine contents per receptacle were 7, 61, 117, 220, and 477 nmol, 0, 5, 10, 15, and 25 d after pollination, respectively. In general, the polyamine concentration in achenes was higher on a fresh weight basis than that of receptacle tissue, and of the three polyamines in achenes, SPD levels were highest (Fig. 3B). As in the case of receptacles, total polyamine levels in achenes were highest (891 nmol/g fresh weight) on the day of pollination; however, unlike receptacles, achenes exhibited a second peak of total polyamines 15 d after pollination, before declining to a minimum (203 nmol/g fresh weight) in ripe fruit.

Effect of auxin on receptacle growth and polyamine levels. Receptacle growth was inhibited by removal of achenes 10 d after pollination. Previous results indicate that the highest concentrations of free IAA in achenes are present at approximately this stage of development, and that free IAA may either be absent, or present only in trace amounts in receptacles during this period (Nitsch, 1950; Dreher and Poovaiah, 1982). Application of α-NAA to the surface of de-achedened receptacles caused a reversal of this growth inhibition (Fig. 4A). Whereas the total polyamine content per receptacle in untreated and auxin-treated receptacles increased during development to a maximum at 20 d after pollination, the polyamine content of de-achedened, auxin-depleted (control) receptacles remained fairly constant (Fig. 4B). One day after removal of achenes, polyamine levels increased dramatically in auxin-treated receptacle tissue when compared with control receptacles. Polyamine levels in control receptacles 24 h after treatment, and in untreated receptacles harvested 10 d after pollination were similar. Subsequent
Fig. 4A,B. Effect of α-naphthaleneacetic acid (α-NAA) on (A) growth and (B) polyamine (PA) content of strawberry receptacles. A. Untreated receptacles were allowed to develop normally with intact achenes (▲). Control (●) and NAA-treated receptacles (■) were de-achedened 10 d after pollination and treated with lanolin paste lacking auxin, or with lanolin paste containing α-NAA (1 mM), respectively. Each point represents the mean fresh weight of n ≥ 9 receptacles; error bars indicate the SE. B. Total free PA content (PUT + SPD + SPM) per untreated, control, and NAA-treated receptacle. Data points represent the mean for n ≥ 3 receptacles; error bars indicate the SE.
experiments (Table 8, Appendix A) revealed that similar polyamine concentrations were present in untreated and control receptacles 11 d after pollination i.e., one day after achene removal. The auxin-induced increase in total polyamine levels was primarily a consequence of increases in PUT and SPD concentrations (Fig. 5A;5B), and these elevated SPD levels were maintained in auxin-treated receptacles at 15 d after pollination as well. Although untreated receptacles accumulated greater amounts of polyamines per receptacle compared to the control receptacles 5 and 10 d after treatment, polyamine concentrations expressed on a fresh weight basis were similar in control and untreated receptacles at the various stages of development (Fig. 5A;5B;5C). Independent experiments revealed that application of the natural auxin present in strawberries (IAA), in addition to initiating growth of auxin-depleted receptacles, also caused rapid increases in polyamine concentration in a manner similar to NAA (Table 8, Appendix A). In an effort to determine whether the auxin-induced increase in polyamine levels is required for growth of de-achened receptacles, the polyamine biosynthesis inhibitors, DFMO and DFMA (2 mM each) were applied along with α-NAA in lanolin pastes in a subsequent experiment. However, the inhibitors reduced neither auxin-induced growth nor polyamine levels (Table 9; Fig. 9-10, Appendix A).

Studies on response to various auxins showed that α-NAA was highly effective at stimulating growth of de-achened strawberries, whereas β-NAA had either no effect or a weak stimulatory effect on receptacle growth (Mudge et al., 1981; Narayan et al., 1981). Therefore, the effects of these structurally related compounds on polyamine levels and growth of receptacles were compared during the early stages following achene
Fig 5A,B,C. Effect of α-naphthaleneacetic acid (α-NAA) on (A) PUT, (B) SPD, and (C) SPM concentrations in strawberry receptacles. Untreated receptacles were allowed to develop normally with intact achenes. Control and NAA-treated receptacles were de-achedened 10 d after pollination and treated with lanolin paste lacking auxin, or with lanolin paste containing α-NAA (1 mM), respectively. Data points represent the mean for n ≥ 3 receptacles; error bars indicate the SE.
Table 1. Effect of β-NAA and α-NAA on polyamine levels and growth of strawberry receptacles. Untreated receptacles were neither de-achedened nor treated with lanolin; control receptacles were de-achedened approximately 10 d after pollination and treated with lanolin paste lacking auxin. Auxin-treated receptacles were also de-achedened 10 d after pollination and treated with lanolin paste containing auxin at 1 mM.

<table>
<thead>
<tr>
<th>Polyamine concentration</th>
<th>Polyamine content</th>
<th>Receptacle growth</th>
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<tbody>
<tr>
<td></td>
<td>PUT</td>
<td>SPD</td>
</tr>
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<td>Treatment (after treatment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>d</td>
<td>116 ± 14'</td>
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<tr>
<td>β-NAA</td>
<td>1</td>
<td>196 ± 23</td>
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<tr>
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<tr>
<td>β-NAA</td>
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<td>α-NAA</td>
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<tr>
<td>β-NAA</td>
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<tr>
<td>α-NAA</td>
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<tr>
<td>Untreated</td>
</tr>
</tbody>
</table>

'Values represent mean ± SE for n > 3 receptacles; 'Not determined; 'Change in receptacle dimensions 5 d after treatment
removal and auxin application. In agreement with a previous study (Narayan et al., 1981), α-NAA was more effective than β-NAA at inducing growth of de-achened receptacles in two independent experiments (Table 1). Both compounds caused increases in polyamine levels one and 5 d after treatment. In addition to having a greater stimulatory effect on growth, α-NAA also caused a greater increase in polyamine levels (mainly those of PUT and SPD) than β-NAA, both on a fresh weight and per receptacle basis. In these experiments, PUT concentrations in α-NAA-treated receptacles did not decline as rapidly as in the previous experiment (Fig. 5A) between 1 and 5 d after treatment. This difference was perhaps due to variation in the developmental stage of the fruits at the time of treatment, although fruits were of identical chronological age in both experiments.

Effect of polyamine application on growth of de-achened receptacles. In an effort to determine whether polyamines are capable of substituting for auxin in the induction of receptacle growth, polyamines were applied to the surface of de-achened receptacles. Application of PUT or SPD (1 mM) in lanolin pastes 5 or 10 d after pollination did not induce growth of de-achened receptacles (Table 2). Similarly, a subsequent experiment revealed that application of a solution of PUT, SPD, and SPM (10 mM each) to de-achened receptacles did not initiate growth. Increasing the concentration of the polyamine mixture (100 mM each of PUT, SPD, and SPM) caused browning and dessication of the tissue, and further inhibition of receptacle growth, indicating that polyamines were not able to substitute for NAA under these conditions.
Table 2. Effect of polyamine application on growth of de-achened receptacles. Untreated receptacles were neither de-achened nor treated with lanolin/solution; control receptacles were de-achened and treated with solution lacking auxin and polyamines. Polyamines or auxin were supplied to de-achened receptacles either in the form of a lanolin paste or in solution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fruit/receptacle age</th>
<th>Fruit/receptacle diameter</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>at time of achene removal</td>
<td>at harvest</td>
</tr>
<tr>
<td></td>
<td>days after pollination</td>
<td>mm</td>
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<tr>
<td>Application of compounds in lanolin pastes</td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>—</td>
<td>30</td>
</tr>
<tr>
<td>PUT (1 mM)</td>
<td>5</td>
<td>30</td>
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<tr>
<td>SPD (1 mM)</td>
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<td>PUT (1 mM)</td>
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<tr>
<td>SPD (1 mM)</td>
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<td>30</td>
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<tr>
<td>Application of compounds in solutions*</td>
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<td></td>
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<tr>
<td>Untreated</td>
<td>—</td>
<td>25</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>α-NAA (1 mM)</td>
<td>10</td>
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</tr>
<tr>
<td>PUT; SPD; SPM (10 mM each)</td>
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<td>25</td>
</tr>
<tr>
<td>PUT; SPD; SPM (100 mM each)</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

*Values represent the mean ± SE for n = 3 receptacles (lanolin experiment) or n = 7 receptacles (applications of compounds in solutions); *Various test compounds were applied in solutions of 10 mM sodium phosphate pH 7; 2% DMSO; 0.1% Tween 80. Immediately following removal of achenes 40 μl of test solution was applied to each de-achened receptacle, and additional 20 μl aliquots were applied 5 and 10 d later.
DISCUSSION

The polyamines PUT, SPD, and SPM, identified in both strawberry receptacles and achenes, have previously been found in free form in fruits of other species including apple, mandarin, and pear (Biasi et al., 1988; Nathan et al., 1984; Toumadje and Richardson, 1988). As in the case of strawberry fruits, these polyamines are found at higher concentrations early in development, prior to declining to lower levels in mature fruit. This trend, involving high polyamine levels in tissues undergoing active growth, and lower levels in mature tissues has been described previously in plants (Palavan and Galston, 1982). Growth in many fruits is characterized by a cell division phase followed by a period of during which cell expansion, but not cell division, occurs. The occurrence of high polyamine concentrations during the early stages of fruit development, and studies which demonstrate that polyamines are needed for normal cell division and growth of bacteria and animal tissues (Tabor and Tabor, 1984), have given rise to the idea that polyamines may be important for cell division in fruits as well. Therefore, high polyamine concentrations during the early stages of strawberry receptacle development may reflect their importance for cell division.

Polyamine concentrations remained consistently higher in achenes than in receptacles, and the relative proportions of polyamines in achenes during development also differed from those of receptacles. The high levels of SPD in achene tissue, and the two peaks in total polyamine levels (on the day of pollination, and 15 d after pollination), bear a strong similarity to the polyamine profile of potato ovules during postpollination.
fruit development. In the case of potato ovules, elevated polyamine levels were observed at times of increased mitotic activity and differentiation (Olson and Nowak, 1988). Although anatomical details of strawberry achene development were not investigated, it seems likely that high polyamine levels in achenes also correspond to periods of increased cell division and differentiation. In achenes, the change from free to cellular endosperm occurred between 10 and 14 d after anthesis (Thompson, 1963).

Attempts have been made to inhibit polyamine biosynthesis in tobacco and tomato fruits by using DFMA and DFMO, which are irreversible inhibitors of arginine decarboxylase and ornithine decarboxylase, respectively. Inhibition of ornithine decarboxylase activity in tobacco ovaries by addition of DFMO and DFMA, was accompanied by decreases in both polyamine concentrations and growth; however, addition of exogenous PUT along with the inhibitors only partially reversed the inhibition of growth (Slocum and Galston, 1985). In tomato fruits, treatment with DFMO inhibited ornithine decarboxylase activity, and also resulted in a reduction in fresh weight that was reversed by simultaneous application of PUT along with DFMO (Cohen et al., 1982). Subsequent studies revealed that DFMO, in addition to inhibiting growth, also caused a reduction in cell number that was also reversible by exogenous PUT (Teitel et al., 1985). However, a more recent report raises some interesting questions about the effects of DFMO on tomato fruit growth. DFMO-treatment at anthesis, which strongly reduced growth of pollinated tomato ovaries (73% inhibition), was not as effective at reducing growth of auxin-induced ovaries (27% inhibition) despite causing a significant inhibition in ornithine decarboxylase activity (Egea-Cortines et al., 1990). Possible explanations
provided by the authors are that DFMO is a less effective inhibitor of growth that is induced by exogenous auxins, or that greater inhibition of pollinated ovaries by DFMO may be a result of higher sensitivity of seeds to the inhibitor. The latter possibility seems to be particularly interesting because of the ability of polyamine biosynthetic inhibitors to reduce somatic embryogenesis. For example, somatic embryogenesis in carrot cultures can be inhibited by DFMA (Feirer et al., 1984). Similarly, DFMA and DFMO, either individually or in combination, proved to be potent inhibitors of somatic embryogenesis in *Hevea brasiliensis* (El Hadrami and D’Auzac, 1992). In addition, we have observed that application of a combination of DFMO and DFMA (5 mM each in 10 mM sodium phosphate, pH 7) to the surface of developing strawberry fruits (with intact achenes) caused rapid browning of the achenes and overall reduction of fruit growth (unpublished observations). Therefore, the inhibitory effect of compounds such as DFMO and DFMA on fruit growth could be the result of an inhibition of zygotic embryogenesis and/or seed development, which in turn may result in decreased synthesis and export of hormones from seeds to the fruit.

Unlike free auxin, which appears to be concentrated primarily in strawberry achenes approximately 10 d after pollination (Dreher and Poovaiah, 1982), substantial amounts of polyamines are present in both achene and receptacle tissue (Fig. 3). Further, removal of achenes, which causes an inhibition of receptacle growth, did not result in an immediate decrease in receptacle polyamine content. Thus, similar levels of polyamines were found in untreated and de-achened (control) receptacles within one day of achene removal. Since endogenous polyamines, unlike free auxin, do not appear to
be limiting at this stage of growth, it is not surprising that exogenous application of polyamines to de-acheded receptacles failed to induce growth. In contrast, in dormant tubers of *Helianthus tuberosus*, where endogenous polyamines were not detected and were presumably limiting, exogenous polyamines were capable of substituting for auxin in the induction of growth (Bagni, 1966). Thus, the similarity in polyamine concentrations of untreated and control receptacles suggests that polyamines do not play an auxin-like role in regulating receptacle growth.

Application of auxin to de-acheded receptacles causes a rapid elevation of PUT and SPD levels, but this increase is probably not necessary for normal receptacle growth, because untreated (normally developing) receptacles do not exhibit increases in polyamine concentrations at the same times as de-acheded, auxin-treated receptacles. However, since achenes may also be a rich source of other plant hormones (Lis et al., 1978), auxin-treated receptacles devoid of achenes may have an altered hormonal balance and different growth requirements than untreated receptacles with intact achenes. Hence, the rapid elevation in polyamine levels following auxin application could be necessary for auxin-induced growth. Consistent with this possibility, the weak auxin, \(\beta\)-NAA, does not cause polyamine levels to increase to the same extent as the more effective auxin, \(\alpha\)-NAA. Since simultaneous addition of polyamine biosynthetic inhibitors and auxin to de-acheded receptacles did not inhibit polyamine accumulation, it remains unclear as to whether the observed increase in polyamine levels is necessary for auxin-induced growth, is merely a consequence of growth, or is totally unrelated to growth. Despite the inability of polyamines to initiate growth, the higher polyamine content per untreated or
auxin-treated receptacle 10 d after treatment may reflect the importance of these compounds for continued development.

The involvement of polyamines in auxin-induced growth has been investigated in processes other than fruit development. The results presented here (Fig. 4B), which demonstrate that auxin is capable of regulating polyamine levels within a short time after treatment, are similar to results described previously in Helianthus tuberosus and mung bean (Friedman et al., 1982; Serafini-Fracassini et al., 1980). In early studies, polyamines were found to substitute for auxin in the initiation of cell division in explants from dormant tubers of H. tuberosus (Bertossi et al., 1965; Bagni, 1966). Subsequent studies revealed that following the auxin-induced break in dormancy of H. tuberosus tubers, levels of PUT, SPD, and SPM increased rapidly (Serafini-Fracassini et al., 1980). In a different cultivar of H. tuberosus, although PUT and SPM were not detected, levels of diaminopropane, cadaverine, and SPD increased following activation of dormant tubers (Phillips et al., 1987). In this study, Phillips et al. (1987) also reported that SPD stimulated cell division in the absence of auxin, but the effect of SPD was small in relation to auxin-stimulated responses. Auxin also increased polyamine levels and stimulated rooting of mung bean hypocotyl cuttings in comparison with control cuttings (Friedman et al., 1982). Unlike auxin treatment, exogenous supply of polyamines to mung bean cuttings in this study did not stimulate rooting (Friedman et al., 1982). However using different rooting conditions, Jarvis et al. (1983) reported that SPM increased both root development and root growth in mung bean hypocotyl cuttings.

In contrast to the stimulatory effect of auxin on polyamine accumulation in
strawberry receptacles, a different effect of auxin on polyamine metabolism has been described in embryogenic tissue cultures, which are induced to differentiate on auxin-containing media, and subsequently undergo differentiation into embryos on auxin-free media. In carrot cultures, transfer of cells to auxin-free media resulted in increased PUT and SPD levels (Montague et al., 1978). However, this effect may not be universal because certain embryogenic alfalfa lines accumulated PUT during the induction period on auxin-containing media, and exhibited lower PUT levels upon transfer to auxin-free media (Meijer and Simmonds, 1988).

In summary, the strawberry fruit represents a system wherein auxin regulates growth and polyamine levels. The rapid increase in polyamine levels following application of auxin to de-achened receptacles is intriguing, although its importance for receptacle growth remains unclear. At the concentrations tested, polyamines were unable to substitute for auxin in the induction of growth of de-achened receptacles, implying that they may not have a hormonal role in this system. Since auxins can initiate parthenocarpic fruit development in strawberries, in addition to being involved in the later stages of growth, further investigation of polyamine metabolism in the strawberry may offer greater insight into possible roles of polyamines at various stages of fruit development.
LITERATURE CITED


Bohner, J. and Bangerth, F. 1988. Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. Physiol. Plant. 72:316-320


Mizrahi, Y. and Heimer, Y.M. 1982. Increased activity of ornithine decarboxylase in tomato ovaries induced by auxin. Physiol. Plant. 54:367-368


CHAPTER II

VACUUM INFILTRATION OF POLYAMINES INCREASES FIRMNESS OF STRAWBERRY SLICES UNDER VARIOUS STORAGE CONDITIONS

Abstract

Strawberry slices were vacuum infiltrated with polyamines and changes in firmness during storage were determined. Spermine and spermidine at 10 mM or 100 mM significantly increased firmness, whereas putrescine was less effective at increasing firmness of slices stored at 1 °C. The firming effect of polyamines was similar to that of calcium chloride, and may have been due to their ability to bind to cell walls and membranes. In ripe receptacles of various cultivars which differed in firmness, putrescine (12-38 nmol/g fresh weight) and spermidine (19-33 nmol/g fresh weight) were the predominant polyamines, while only low levels of spermine (< 10 nmol/g fresh weight) were present. There was no discernible relationship between endogenous polyamine levels and fresh fruit firmness for the cultivars studied.
INTRODUCTION

Polyamines are present in plants, animals and microorganisms (Evans and Malmberg, 1989; Galston, 1983). In plants, the polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) have been implicated in a number of physiological processes such as cell division, cell expansion, flowering, fruit development and ripening, response to stress, and senescence (Evans and Malmberg, 1989; Galston, 1983; Smith, 1985).

Polyamines may also influence the postharvest physiology of fruits (Kramer and Wang, 1989; Toumadje and Richardson, 1988). These compounds are thought to have an anti-senescent effect on plants (Altman et al., 1977), and may have an inhibitory effect on the biosynthesis of the plant hormone ethylene (Apelbaum et al., 1981; Ke and Romani, 1988). Elevated endogenous levels of polyamines were also correlated with prolonged keeping qualities of certain tomato cultivars (Dibble et al., 1988; Saftner and Baldi, 1990). Similarly, during controlled atmosphere storage, reduced rates of softening were correlated with increased levels of endogenous polyamines in apples (Kramer et al., 1989). There is also evidence that direct application of polyamines can have beneficial effects on the storage life of fruits. Infiltration with SPM prior to storage at 2.5 °C, caused a reduction of chilling injury in zucchini squash (Kramer and Wang, 1989). Pressure infiltration of polyamines caused an immediate increase in apple fruit firmness and a decreased rate of softening at 0 °C (Kramer et al., 1991). In addition, vacuum infiltration of polyamines into tomatoes increased the shelf life of the polyamine-treated
fruit (Davies et al., 1990).

Polyamines are positively charged and have similar properties to calcium in terms of their ability to bind isolated fruit pectin (D'Orazi and Bagni, 1987), delay senescence, and retard fruit softening (Kramer et al., 1991). Since calcium treatment effectively reduced postharvest softening of strawberries (Main et al., 1986; Rosen and Kader, 1989), polyamines may have a similar effect on maintaining strawberry fruit firmness.

Based on the evidence suggesting that polyamines influence the postharvest physiology of fruits, this study was conducted to determine if the softening of strawberry slices could be retarded by treatment with these naturally occurring compounds. In addition, PUT, SPD, and SPM were identified and quantitated in various cultivars of strawberry that differed in firmness to determine if there was any relationship between polyamine content and firmness of fresh fruit.

MATERIALS AND METHODS

Chemicals

Polyamines (hydrochloride salts) were purchased from Sigma Chemical Co., St. Louis, MO. Calcium chloride·2H$_2$O was obtained from Fisher Scientific, Pittsburgh, PA. Ronilan WP [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione] was purchased from BASF Corp., Parsippany, NJ. All other chemicals were ACS reagent grade. The solutions for vacuum infiltration were prepared by dissolving the various compounds in deionized distilled water and adjusting to pH 7.0 with NaOH or HCl.
Control slices were vacuum-infiltrated with deionized distilled water (pH 7). Phosphate buffer (pH 7) was prepared by mixing appropriate amounts of monobasic sodium phosphate with dibasic sodium phosphate.

**Plant material**

Strawberries (*Fragaria x ananassa* Duch.) were obtained either from research plots at OARDC or from a private farm in Wooster, OH. Storage experiments were conducted with ripe fruit (cultivar Lateglow) obtained during the 1990, 1991, and 1992 growing seasons. Damaged fruits were discarded and the remaining fruits were rinsed with water and allowed to drain on a mesh screen. After the calyx was removed, a longitudinal slice was cut through the center of each receptacle using a knife with two blades set 1 cm apart, the peripheral slices were discarded, and all further studies were concerned with the 1 cm wide central section.

Vacuum infiltration was carried out by placing about 25 slices in 125 ml of test solution in a plastic container (11 cm x 11 cm x 4 cm) and applying a vacuum of 127 mm of Hg for 8 min. For studies involving storage of slices, test solutions were drained, slices were placed in 100 x 15 mm polystyrene petri-dishes (5 slices/dish), sealed with parafilm and placed in the dark at the desired temperature. For all 1991 storage experiments (Fig 6; Tables 4-5), slices were dipped in Ronilan fungicide (1.2 g/l) immediately after vacuum infiltration for control of *Botrytis cinerea* development; untreated slices were also dipped in Ronilan before firmness measurement or storage. Slices were not treated with fungicide in experiments conducted during 1990 and 1992.
**Firmness measurements**

Firmness was measured using an Instron (1011) fitted with a 3.2 mm probe (Instrument settings: compression; 500 g load range; 50 mm/min crosshead speed). Following storage, the tissue was allowed to equilibrate at room temperature, and the maximum reading in grams was recorded during the passage of the probe through the 1 cm wide receptacle section. Unstored slices were vacuum infiltrated, drained, and firmness was measured about 30 min after vacuum infiltration. Two such peak firmness measurements were made on the cortical region of each slice, one on either side of the central pith area. The mean of these two measurements constituted the firmness value for each slice.

**Polyamine analysis**

Following firmness measurements, 10 freshly prepared receptacle slices from ripe fruits of each of 9 cultivars were frozen individually in liquid nitrogen and stored at -20 °C. Each slice (i.e., 10 reps./cultivar) was analyzed individually for polyamines. Achenes were removed from the frozen receptacle surfaces and the slices were homogenized in 5% perchloric acid (5 ml perchloric acid solution per gram fresh weight of tissue). The homogenate was incubated for 1 h on ice, then centrifuged at 16,000 xg for 25 min at 4 °C. The supernatant was then analyzed for free polyamines. Polyamines in the perchloric acid extract (0.5 ml) were benzoylated, then separated and quantitated by HPLC according to Slocum et al. (1989) using gradient elution program 1. Chromatography was performed using an IBM (LC/9533) Ternary Gradient Liquid
RESULTS AND DISCUSSION

Initial experiments were conducted in 1990 (Table 3) to determine the effects of vacuum infiltration of polyamines on firmness of strawberry slices obtained from two different harvests of 'Lateglo\textregistered', a relatively soft-fruiting cultivar (Scheerens and Brenneman, 1991). Following storage, untreated and calcium-treated slices exhibited the least amount of visible surface damage. Slices vacuum infiltrated with water (control) appeared to be damaged to the greatest extent, exhibiting a soft, water-soaked appearance, and polyamine-infiltrated slices exhibited less surface damage than the control. Calcium treatment resulted in slices that were lighter in color than all other slices, and had a firmer surface appearance. In addition to causing a damaged appearance, polyamine treatment resulted in a greater amount of microbial growth when compared with other treatments. However, firmness measurements revealed that following storage, 100 mM SPD-treated slices were firmer than both untreated and control slices, but were not always as firm as 100 mM calcium-treated slices (Table 3). Lower concentrations of SPD (10 mM) were also effective at increasing firmness when compared to the control. PUT-treated slices were firmer than control slices, but the difference was not statistically significant in Experiment II. PUT was less effective than SPD or calcium at increasing firmness, and untreated slices were similar in firmness to Chromatograph with an IBM UV (254 nm) detector and a C-18 column (IBM; 4.5 x 250 mm).
Table 3. Firmness of ‘Lateglow’ strawberry slices after vacuum infiltration and storage in 1990.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>45 b</td>
<td>45 c</td>
</tr>
<tr>
<td>Control (water)</td>
<td>26 c</td>
<td>38 c</td>
</tr>
<tr>
<td>CaCl₂ (100 mM)</td>
<td>84 a</td>
<td>92 a</td>
</tr>
<tr>
<td>PUT (10 mM)</td>
<td>44 b</td>
<td>49 c</td>
</tr>
<tr>
<td>PUT (100 mM)</td>
<td>50 b</td>
<td>52 c</td>
</tr>
<tr>
<td>SPD (10 mM)</td>
<td>59 b</td>
<td>75 b</td>
</tr>
<tr>
<td>SPD (100 mM)</td>
<td>80 a</td>
<td>71 b</td>
</tr>
</tbody>
</table>

*Slices were stored for 8 d at 1 °C followed by 2 d at room temperature (Experiment I); or for 9 d at 1 °C followed by 1 d at room temperature (Experiment II); *Mean separation in the same column by Duncan’s multiple range test (p ≤ 0.05). Means in columns followed by the same letter(s) are not significantly different (n ≥ 20 slices).
PUT-infiltrated slices. Water infiltration caused softening of the slices when compared to untreated slices, although this difference was not significant in Experiment II (Table 3).

The following year (1991), experiments were conducted to re-examine the effects of infiltration of individual polyamines on the firmness of stored fruits and to determine the influence of polyamine concentration, storage condition, and time on firmness. The compounds which had the greatest firming effect (SPD and calcium chloride) in experiments conducted during the previous year, were vacuum infiltrated into slices and changes in firmness over time were determined (Fig. 6). Immediately after vacuum infiltration, firmness was similar in control, SPD, and calcium-treated slices. However, after 3 d of storage at 1 °C, both SPD and calcium-treated slices were firmer than control slices, and this increased firmness was maintained over the 18 d storage period (Fig. 6). Similarly, Rosen and Kader (1989) found no difference in fruit firmness on the day of treatment following dip treatments in water or calcium chloride. However, after storage at 2.5 °C for 7 d, calcium-treated slices were significantly firmer than water-treated slices.

In another experiment conducted in 1991, firmness of slices vacuum-infiltrated with polyamines or calcium (at 100 mM) was compared to control and untreated slices both before storage and after storage under different conditions (Table 4). There was no significant difference in firmness of slices immediately after vacuum infiltration with various compounds. Storage condition had a substantial effect on the firmness of all treatments examined. Following storage of slices for 9 d at 1 °C, calcium, SPD, and
Fig. 6. Firmness of vacuum-infiltrated 'Lateglow' strawberry slices (1991) during storage at 1 °C. Slices were vacuum infiltrated with water (○), 100 mM calcium chloride (●), or 100 mM SPD (▲), then dipped in Ronilan. Prior to storage and at specified times after storage at 1 °C, firmness was determined, and slices were discarded immediately after firmness measurements. Each point represents the mean ± SE for n \geq 20 slices.
Table 4. Firmness of vacuum-infiltrated ‘Lateglow’ strawberry slices in 1991, prior to storage, or following storage for either 4 d at 20 °C, or 9 d at 1 °C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Firmness (g)</th>
<th>Day 4 (20 °C)</th>
<th>Day 9 (1°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>44a†</td>
<td></td>
<td>39 ab</td>
<td>43 bcd</td>
</tr>
<tr>
<td>Control (water)</td>
<td>47a</td>
<td></td>
<td>11 b</td>
<td>27 d</td>
</tr>
<tr>
<td>CaCl₂ (100 mM)</td>
<td>44a</td>
<td></td>
<td>60 a</td>
<td>64 a</td>
</tr>
<tr>
<td>PUT (100 mM)</td>
<td>45a</td>
<td></td>
<td>23 b</td>
<td>41 cd</td>
</tr>
<tr>
<td>SPD (100 mM)</td>
<td>44a</td>
<td></td>
<td>33 ab</td>
<td>63 ab</td>
</tr>
<tr>
<td>SPM (100 mM)</td>
<td>38a</td>
<td></td>
<td>35 ab</td>
<td>54 abc</td>
</tr>
</tbody>
</table>

†Mean separation in the same column by Duncan's multiple range test (p ≤ 0.05). Means in columns followed by the same letter(s) are not significantly different (n ≥ 5 slices).
SPM treatments caused an increase in firmness compared to the control. In addition, calcium-treated slices were significantly firmer than untreated and PUT-treated slices, thus calcium was (at 100 mM) the most effective compound at increasing firmness, regardless of storage condition or growing season. Storage for 4 d at 20 °C resulted in similar firmness of control, untreated, or polyamine-treated slices, whereas calcium-infiltrated slices were firmer than control slices (Table 4). Softening of all slices at 20 °C for 4 d could have minimized differences in firmness that may have been evident following a shorter storage period. As part of the same experiment, the effect of different concentrations of polyamines or calcium on firmness was compared (Table 5). Under both storage conditions, the lowest concentration of the compounds tested (1 mM) was less effective at maintaining fruit firmness when compared to higher concentrations (10 mM or 100 mM). Of the polyamines, PUT was the least effective compound at maintaining firmness of slices, whereas SPD and SPM had similar effects on firmness of slices under both storage conditions.

Studies on apple and pear showed that buffers (pH 7-9) can increase firmness of fruit tissue (Knee, 1982). Phosphate buffer at pH 7 (50 mM or 83 mM), which has greater buffering capacity than 10 mM SPD (pH 7) in water (Fig. 14, Appendix B), was less effective at increasing firmness than the polyamine solution (Table 6). Infiltration of slices with solutions of SPD in either water or 50 mM phosphate buffer resulted in slices of similar firmness. These data suggested that SPD increases firmness of strawberry slices not merely by serving as a buffer such as phosphate. Suspensions obtained following homogenization of stored slices did not differ significantly in pH.
Table 5. Firmness of 'Lateglow' strawberry slices (1991) vacuum-infiltrated with different levels of calcium or polyamines, after 4 d storage at 20 °C, or after 9 d storage at 1 °C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 4 (20 °C)</th>
<th>Day 9 (1 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (water)</td>
<td>11 ± 4*</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>CaCl₂ (1 mM)</td>
<td>10 ± 3</td>
<td>33 ± 12</td>
</tr>
<tr>
<td>(10 mM)</td>
<td>22 ± 6</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>(100 mM)</td>
<td>60 ± 19</td>
<td>64 ± 12</td>
</tr>
<tr>
<td>PUT (1 mM)</td>
<td>10 ± 1</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>(10 mM)</td>
<td>14 ± 2</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>(100 mM)</td>
<td>23 ± 6</td>
<td>41 ± 10</td>
</tr>
<tr>
<td>SPD (1 mM)</td>
<td>6 ± 1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>(10 mM)</td>
<td>25 ± 8</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>(100 mM)</td>
<td>33 ± 6</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>SPM (1 mM)</td>
<td>4 ± 1</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>(10 mM)</td>
<td>30 ± 9</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>(100 mM)</td>
<td>35 ± 11</td>
<td>54 ± 3</td>
</tr>
</tbody>
</table>

*Values represent the mean ± standard errors for 5 or more replicate samples
Table 6. Firmness of 'Lateglow' strawberry slices in 1992, following vacuum infiltration with sodium phosphate buffer (pH 7) and SPD, and storage for 10 d at 1 °C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Firmness (g)</th>
<th>pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>45c*</td>
<td>3.62a</td>
</tr>
<tr>
<td>Control (water)</td>
<td>41c</td>
<td>3.63a</td>
</tr>
<tr>
<td>Phosphate (50 mM)</td>
<td>43c</td>
<td>3.66a</td>
</tr>
<tr>
<td>Phosphate (83 mM)</td>
<td>57b</td>
<td>3.69a</td>
</tr>
<tr>
<td>SPD (10 mM) in water</td>
<td>73a</td>
<td>3.52a</td>
</tr>
<tr>
<td>SPD (10 mM) in phosphate (50 mM)</td>
<td>76a</td>
<td>3.76a</td>
</tr>
</tbody>
</table>

*Mean separation in the same column by Duncan's multiple range test (p ≤ 0.05). Means in columns followed by the same letter are not significantly different (n=15 slices for firmness measurements; n=3 slices for pH measurements); *The pH was determined after homogenization of stored slices.
These results showed that polyamines significantly increased firmness of strawberry slices after storage at 1 °C. This firming effect of polyamines was seen in fruit from three different seasons. The increased fruit firmness following polyamine treatment in strawberries is similar to previous studies wherein polyamine infiltration increased firmness of apples (Kramer et al., 1991). Similarly, infiltration with polyamines also caused an increase in the shelf life of tomatoes and resulted in fruits that were firmer than water-infiltrated fruits (Davies et al., 1990; Law et al., 1991).

During ripening, the strawberry fruit softens, the cell wall matrix and middle lamella become hydrated and large amounts of polyuronide in the wall become soluble (Knee et al., 1977). Strawberry fruits have been subjected to various treatments in an effort to increase their shelf life and to improve firmness (Ke et al., 1991; Main et al., 1986). Treatment with calcium has proved to be an effective means for reducing postharvest softening of strawberry fruits and slices (Main et al., 1986; Rosen and Kader, 1989). The effect of calcium in reducing softening is thought to be a result of cross-linking of calcium ions with pectin in the middle lamella (Morris et al., 1985; Neal, 1965). Increased fruit firmness following postharvest calcium treatment has been previously reported in strawberries (Rosen and Kader, 1989). Foliar sprays of calcium, also increased calcium levels and decreased softening of strawberry fruits (Cheour et al., 1991; Cheour et al., 1990). Polyamines are positively charged, and when applied exogenously, may have similar effects to calcium in terms of their ability to delay senescence and retard fruit softening in apples (Kramer et al., 1991). Studies on uptake of polyamines by cells suggested that substantial amounts of the polyamines taken up are
bound to the cell wall (Pistocchi et al., 1987; Scoccianti et al., 1989). In addition, polyamines, like calcium, bind to pectin isolated from fruit tissue (D’Orazi and Bagni, 1987). SPD and SPM show a higher affinity than PUT for isolated polysaccharides (Scoccianti et al., 1989). The greater ability of the triamine SPD, and the tetramine SPM, to bind isolated polysaccharides may partially explain why PUT, a diamine, was less effective at increasing firmness of strawberry slices than SPD or SPM at the same concentrations (Tables 3 and 5). Because of the importance of ionic cross-linking for maintenance of cell wall rigidity, the effect of polyamines on increasing firmness of strawberry slices may be due, in part, to stabilization of cell walls.

In addition to their possible effects on cell walls, polyamines may affect strawberry slices by virtue of their ability to stabilize membranes (Altman et al., 1977). Studies using membrane probes revealed that exogenous application of polyamines to microsomal membranes resulted in a reduction in fluidity at the membrane surface through association of the polyamines with membrane lipid (Roberts et al., 1986). At equal molar concentrations, SPD and SPM were more effective than PUT at reducing membrane viscosity (Roberts et al., 1986). Calcium also increased the rigidity of cell membranes (Paliyath et al., 1984; Poovaiah, 1986; Roberts et al., 1986), thus suggesting again that the ability of calcium and the polyamines to retard softening (Tables 3-5; Kramer et al., 1991) may be due to similar effects on fruit tissue. Although strawberries are non-climacteric, they produce ethylene during postharvest storage (Li and Kader, 1989). The effect of the polyamines on inhibition of strawberry softening could partially be due to an inhibition of wound-induced ethylene synthesis. Another possibility is that
polyamines may inhibit the action of enzymes involved in softening. For instance, Kramer et al. (1989) found polyamines to have an inhibitory effect on polygalacturonase extracted from apples that had been inoculated with fungus. Although polygalacturonase may not be present in strawberries (Barnes and Patchett, 1976; Huber, 1984), polyamines could possibly affect other wall-degrading enzymes in addition to physically stabilizing the tissue. Kramer et al. (1991) speculate that polyamines may maintain fruit quality by stabilizing cell walls, or by making cell walls less accessible to wall-softening enzymes.

The effects of exogenous application of polyamines may not necessarily reflect their true physiological role (Roberts et al., 1986). To determine whether there was any relation between polyamine levels and fresh fruit firmness, endogenous polyamine levels were measured in several cultivars that differ in firmness (Table 7). The polyamines PUT, SPD, and SPM were found in receptacle tissue, while cadaverine was not detected in receptacles of any of the cultivars tested. SPD and PUT were the predominant polyamines while SPM was present at low levels in ripe receptacle tissue. Fresh fruit firmness and levels of either individual or total polyamines in the various cultivars were not correlated \( (p \leq 0.05) \). Endogenous polyamines may influence firmness of strawberry slices during storage, although this possibility was not investigated. In a recent study, vacuum infiltration of polyamines was found to increase storage life of tomato fruits, although the levels of polyamines applied were not substantially greater than the natural levels (Law et al., 1991). It is possible that exogenously applied polyamines reach different sites than endogenous polyamines; therefore the effects of applied polyamines may not reflect the normal physiological role of these compounds.
<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Firmness (g)</th>
<th>PUT</th>
<th>SPD</th>
<th>SPM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parker</td>
<td>108 ± 12</td>
<td>15 ± 2</td>
<td>25 ± 3</td>
<td>3 ± 1</td>
<td>43</td>
</tr>
<tr>
<td>Kent</td>
<td>91 ± 9</td>
<td>17 ± 2</td>
<td>19 ± 1</td>
<td>2 ± 1</td>
<td>38</td>
</tr>
<tr>
<td>Oso Grande</td>
<td>82 ± 9</td>
<td>25 ± 5</td>
<td>29 ± 3</td>
<td>4 ± 2</td>
<td>58</td>
</tr>
<tr>
<td>Allstar</td>
<td>74 ± 6</td>
<td>38 ± 3</td>
<td>32 ± 2</td>
<td>4 ± 1</td>
<td>74</td>
</tr>
<tr>
<td>Canoga</td>
<td>72 ± 3</td>
<td>26 ± 2</td>
<td>30 ± 3</td>
<td>3 ± 1</td>
<td>59</td>
</tr>
<tr>
<td>Micmac</td>
<td>56 ± 3</td>
<td>12 ± 1</td>
<td>21 ± 1</td>
<td>4 ± 1</td>
<td>37</td>
</tr>
<tr>
<td>Lateglow</td>
<td>52 ± 3</td>
<td>18 ± 1</td>
<td>27 ± 2</td>
<td>9 ± 3</td>
<td>54</td>
</tr>
<tr>
<td>Totem</td>
<td>50 ± 4</td>
<td>27 ± 2</td>
<td>26 ± 2</td>
<td>4 ± 1</td>
<td>57</td>
</tr>
<tr>
<td>Catskill</td>
<td>38 ± 3</td>
<td>29 ± 4</td>
<td>33 ± 3</td>
<td>5 ± 3</td>
<td>67</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE for 10 replicate samples
To conclude, vacuum infiltration of polyamines followed by storage at 1 °C increased firmness of strawberry slices. This firming effect of the polyamines was similar to that of calcium chloride and may be due to a stabilizing effect on cell walls and/or membranes. Of the polyamines tested, SPD and SPM had a greater firming effect than PUT. Although endogenous polyamines were found in receptacles of all cultivars tested, there was no relation to fresh fruit firmness. It is not clear if the delayed softening due to polyamine treatment of strawberry slices reflects their normal physiological function. Future studies have been planned to determine whether introduction of polyamines into developing strawberry fruits can improve their keeping qualities.


GENERAL DISCUSSION

Summary of results

Polyamines were identified in strawberry (cultivar Allstar) receptacles and achenes at all stages of fruit development (Chapter I). Diaminopropionate and CAD were not detected in fruit tissue, but PUT, SPD, and SPM were present throughout development, and their levels and proportions fluctuated depending on developmental stage.

A unique characteristic of the strawberry, which permits removal and addition of a hormonal source at specific developmental stages, was exploited for the purpose of investigating a role for polyamines in auxin-induced growth. Unlike auxin concentrations, which decline dramatically following removal of achenes (Archbold and Dennis, 1985), polyamine concentrations remained similar in untreated receptacles (with intact achenes), and de-achedened receptacles. Thus, removal of achenes does not appear to cause polyamine concentrations to be limiting in receptacles. Polyamines also could not substitute for auxins when applied to the surface of de-achedened receptacles, implying that they do not play a hormonal role in this system.

Auxin application to the surface of de-achedened receptacles resulted in rapid elevations in polyamine levels. This response was noted in the case of synthetic auxins and IAA. In de-achedened receptacles, α-NAA caused a greater increase in polyamine levels and growth than β-NAA. Since only de-achedened receptacles treated with auxin
exhibited elevated polyamine concentrations 24 h after treatment, this increase in polyamine levels is probably not a requirement for normal development of untreated receptacles (with intact achenes).

Following achene removal, auxin-treated and untreated receptacles accumulated greater than three times the amount of total polyamines per receptacle than de-achedened receptacles not treated with auxin. Although auxins clearly regulate polyamine levels in de-achedened receptacles, the significance of this hormonally-induced increase in polyamine levels remained unclear. Therefore attempts were made to reduce polyamine levels in de-achedened receptacles by applying inhibitors of polyamine biosynthesis along with auxin. These inhibitors had no effect on receptacle polyamine levels or growth. The ability to specifically inhibit polyamine biosynthesis could potentially provide a better understanding of whether or not these compounds are required for growth. However, significant reduction of polyamine concentrations in a receptacle that is attached to the plant could be difficult to accomplish, especially since the fruit may be capable of importing polyamines from other portions of the plant. When a small quantity of radiolabelled PUT was applied to the surface of a bract or a leaf, movement of the label into the fruit was noted. A substantial amount (about 30%) of label in the fruit was in the form of polyamines (Table 12-13, Appendix A). These data contrast with the view that polyamines may not be translocated (Young and Galston, 1983; Galston, 1983), but are in agreement with the results of Beraud et al. (1992).

In addition to topical application of polyamine biosynthetic inhibitors to the surface of strawberry receptacles, attempts were made to introduce inhibitors into
developing fruits by other methods. When introduced via a cotton wick, a combination of DFMO and DFMA caused inhibition of fruit growth (Table 14, Appendix A). However, simultaneous supply of PUT with the inhibitors did not overcome this inhibition, suggesting that the effect of the inhibitors may not have been specific to polyamine biosynthesis. Achenes appeared to be particularly sensitive to the inhibitors, since they turned dark brown to black in color when they came into contact with the inhibitor solutions. Receptacle growth was inhibited in the area associated with these darkened achenes. Attempts were also made to culture ‘Allstar’ fruits in vitro with the aim of introducing inhibitor solutions into fruits. Significant growth of excised ‘Pajaro’ fruits cultured approximately 12 d after anthesis, was reported by Perkins-Veazie and Huber (1992). ‘Allstar’ fruits grew to a limited extent when cultured in vitro, thus making it difficult to evaluate the effects of polyamine biosynthetic inhibitors on growth (Table 15, Appendix A). The limited growth of cultured fruits reported here (Table 15, Appendix A), in comparison with the growth of ‘Pajaro’ fruits (Perkins-Veazie and Huber, 1992) could be due to the difference in cultivars studied, since fruits of ‘Douglas’ did not develop when cultured in vitro (Perkins-Veazie and Huber, 1992).

A subsequent study was conducted to determine the influence of polyamine treatment on firmness of strawberry fruit slices (Chapter II). A relatively soft-fruited cultivar, Lateglow, was used in experiments conducted with fruits obtained during three growing seasons. Vacuum infiltration of polyamines increased firmness of slices stored under various conditions. In general, SPD and SPM (10 or 100 mM) were more effective at increasing firmness than PUT at similar concentrations. Because of the
importance of cations for maintenance of cell wall structure of strawberries (Neal, 1965), and the ability of polyamines to bind to isolated polysaccharides (D'Orazi and Bagni, 1987), plant cell walls (Pistocchi et al., 1987), and membranes (Roberts et al., 1986), a major effect of polyamine-infiltration on fruit firmness could be due to the stabilization of cell walls and membranes. There was no apparent relationship between fresh fruit firmness and endogenous polyamine concentration in receptacles of nine cultivars tested.

Problems encountered and possible solutions

As an experimental system to study polyamines, the strawberry does provide advantages in terms of the ability to inhibit and re-initiate growth at various stages of development, and the relatively rapid rate of fruit development. However, certain difficulties were encountered during the course of this study. As discussed below, many of these difficulties may not be unique to strawberries.

The apparent lack of specificity of assays for polyamine biosynthetic enzymes has been a problem. Conventional assays of key polyamine biosynthetic enzymes such as ornithine decarboxylase and arginine decarboxylase involve trapping of the $^{14}$CO$_2$ released following incubation of cell-free extracts with radiolabelled substrates (Biasi et al., 1991; Slocum and Galston, 1985a). Preliminary experiments revealed high pH optima for decarboxylation of ornithine and arginine by strawberry extracts. Although the reaction products were not analyzed in this study, other workers have shown that high pH optima may indicate non-enzymatic decarboxylation of amino acids by plant extracts (Smith and Marshall, 1988 a,b). Therefore for strawberry extracts, the use of labelled amino acids
for the measurement of enzymatic activity, followed by chromatographic separation of labelled product may be a more prudent approach than the use of a $^{14}$CO$_2$-trapping assay. For instance, to assay ornithine decarboxylase activity, dansylation of the reaction products, followed by separation of labelled PUT on silica gel TLC plates, may be a suitable alternative to the $^{14}$CO$_2$-trapping assay. The method of Birecka et al. (1985 a,b), which entails the use of thin-layer electrophoresis of labelled reaction products for the measurement of decarboxylase activity in plant extracts, would also be more specific than the $^{14}$CO$_2$-trapping assay. A reliable method for measurement of enzyme activity could reveal the relative importance of arginine decarboxylase and ornithine decarboxylase for PUT synthesis during strawberry fruit development.

Observation of the effects of polyamine depletion on fruit development could provide valuable information on the function of these compounds. An approach that has been attempted with varying degrees of success involves the use of enzyme-activated irreversible inhibitors of polyamine biosynthetic enzymes. These inhibitors are valuable tools provided they specifically inhibit the target enzyme, and supply of the limiting metabolite reverses the effect of the inhibitor. Topical application of DFMO and DFMA to strawberry receptacles did not reduce growth or polyamine levels. Similarly, application of DFMO and DFMA to apple fruits did not inhibit growth (Miller et al., 1988). In tobacco, when flowers were excised at anthesis and treated with solutions of DFMO or DFMA via the cut end of the peduncle, ovary growth was inhibited 32% or 52%, respectively, in comparison with the buffer-treated control (Slocum and Galston, 1985b). A treatment of DFMA + DFMO caused slightly greater growth inhibition (63%)
than either inhibitor alone, but simultaneous supply of PUT only partially reversed this effect so that growth was inhibited 45% in the DFMA+DFMO+PUT-treatment. Since ornithine decarboxylase activity was much greater than that of arginine decarboxylase, an interesting observation made in this study of tobacco ovary growth was that DFMA-mediated inhibition of extractable ornithine decarboxylase activity was probably due to arginase-mediated conversion of DFMA to DFMO. Application of DFMO by the split stem technique reduced growth and ornithine decarboxylase activity of pollinated tomato fruits, and simultaneous supply of PUT reversed this inhibition (Cohen et al., 1982). These data suggest an important role for ornithine decarboxylase and PUT in tomato fruit development. Curiously, DFMO did not inhibit growth or cell division of parthenocarpic (auxin-induced) fruits in a subsequent study (Egea-Cortines and Mizrahi, 1993), which implies that the inhibitor may be reducing growth of pollinated tomato fruits by affecting seed development. The results of many of the studies discussed above indicate that specific inhibition of polyamine biosynthesis has been difficult to achieve in intact fruits.

In addition to conventional approaches involving the use of inhibitors for the study of polyamines in plants, molecular approaches are beginning to emerge (Bell and Malmberg, 1990; Hammill et al., 1990). Studies incorporating the use of molecular techniques, along with the physiological methods that are presently employed, are likely to result in a better understanding of polyamine function in plants.

Little is known about the distribution of polyamines within the cell. A key factor in understanding a role for polyamines in both fruit and whole plant development could be knowledge about their subcellular locations. Despite the potential benefits resulting
from polyamine application, it is not known if exogenously applied polyamines occupy the same sites as their endogenous counterparts. Although analysis of receptacle tissue revealed similar polyamine concentrations in de-achened and untreated receptacles (Chapter I), it is possible that their subcellular locations varied depending on the presence of auxin. Calcium, which may be a hormonal second messenger, is thought to be present at low concentrations in the cytosol (Brummell and Hall, 1987). Calcium could be released from subcellular locations such as the vacuole (Schumaker and Sze, 1987) following hormonal stimulus, and the transient increase in cytosolic calcium concentration may initiate subsequent processes that result in growth (Brummell and Hall, 1987). Thus, information about polyamine levels in various subcellular locations could be an important factor in understanding the interaction of these compounds with auxins and other hormones.

Polyamines and plant hormones

Bagni and co-workers have proposed that polyamines are plant growth substances or plant hormones in higher plants (Bagni, 1989; Biasi et al., 1991). In general, hormones are present at low concentrations and are effective at internal concentrations of 1 μM or less (Salisbury and Ross, 1978). Endogenous polyamines in fruit tissues are frequently present at concentrations of several hundred nmol/g fresh weight (Chapter I; Biasi et al. 1991; Olson et al. 1988; Winer and Apelbaum 1986). In contrast, auxin levels in citrus, grape, and tomato fruit tissues are often below 1 nmol/g fresh weight and seldom exceed 10 nmol/g fresh weight (Leopold and Kriedemann, 1975; Hocher et al.
According to one study (Dreher and Poovaiah, 1982), the maximum free IAA content per strawberry fruit was 93 ng (or 0.53 nmol). Lis et al. (1978) estimated peak IAA concentrations per gram fresh weight to be approximately 4 μg (23 nmol) for achenes and 0.4 μg (2.3 nmol) for receptacles. Clearly, endogenous polyamine levels are considerably higher than those customarily associated with the accepted hormones.

In some studies, exogenous polyamines have been shown to influence fruit development. The effective polyamine concentrations in many of these studies are quite high. For example, sprays of 50 mM PUT dichloride or 0.5-1.0 M PUT base increased fruit set of olive, but at lower concentrations (0.5 mM) PUT did not affect fruit set (Rugini and Mencuccini, 1985). In apples, the effects of polyamine sprays varied in different studies. Costa and Bagni (1983) reported that spraying apple trees with low concentrations of polyamines (1 to 100 μM) enhanced fruit set, yield per tree, and fruit growth. This effect is apparently not universal since Volz and Knight (1986) and Miller et al. (1988) did not observe any beneficial effects as a result of polyamine sprays on apple trees. The high concentrations of polyamines required to improve fruit set of olive (Rugini and Mencuccini, 1985), inconsistent results with different studies on apple, and the lack of effect of applied polyamines on growth of de-achened receptacles, provide insufficient evidence for classifying these compounds as plant hormones during fruit set and growth.

Even though polyamines do not appear to carry out a hormonal function in plants, the possibility that they mediate the effects of plant hormones in some systems cannot be excluded. Polyamines have been shown to substitute for auxins in inducing development,
but this effect usually requires higher concentrations of polyamines than auxin, and the applied polyamines are often less effective than auxins at inducing development (Jarvis et al. 1983; Phillips et al., 1987). In strawberry, exogenous polyamines did not substitute for auxin in inducing growth of de-achened receptacles (Chapter I). Hormones are capable of inducing rapid changes in polyamine levels (Chapter I; Serafini Fracassini et al., 1980), often prior to growth. However, the inability to specifically reduce polyamine levels makes it difficult to determine whether polyamines are hormonal second messengers in plants, or if changes in their levels constitute a non-specific response unrelated to growth.

**Polyamines in postharvest physiology**

Studies of the possible involvement of polyamines in postharvest physiology have yielded interesting results both from measurements of endogenous polyamine levels and from polyamine application. Tomato fruits from cultivars that had better keeping qualities, also had higher endogenous polyamine concentrations than more rapidly ripening cultivars (Dibble et al., 1988; Saftner and Baldi, 1990). Introduction of polyamines and their precursors into tomato fruits also increased the shelf life of treated fruits (Davies et al. 1990; Law et al. 1991). In apple, controlled atmosphere storage reduced the rate of softening and resulted in elevated polyamine levels (Kramer et al., 1989). Infiltration of polyamine solutions also increased firmness of apples (Kramer et al., 1991). Changes in firmness as a result of polyamine application are not restricted to climacteric fruits such as apple and tomato, since infiltration of polyamines increased
firmness of stored strawberry slices (Chapter II). Fresh fruit firmness of strawberry receptacles was not correlated with endogenous polyamine concentration in different cultivars. Fruit firmness is probably affected by many factors including degree of methylation of pectin and calcium content (Neal, 1965). If polyamines are important for fruit firmness, they may be one of many contributing factors, and are unlikely to be the sole factor involved.

Conclusion

Polyamines do not seem to have a hormonal function in strawberries and other fruits. However, their levels change significantly in response to developmental stage and auxin-treatment. Exogenous polyamine application can have beneficial effects on fruit set and postharvest physiology. The ability to specifically reduce polyamine levels in fruits would facilitate interpretation of their role in development. For example, specific inhibition of polyamine biosynthesis in whole plants by application of polyamine biosynthetic inhibitors such as DFMO to the soil, caused inhibition of growth that was reversed by supplying PUT (Burtin et al., 1991). Studies with cell cultures indicate that depletion of polyamines caused inhibition of growth that could be overcome by supplying the limiting metabolite (Minocha et al., 1991; Ponappa et al., 1992; Tabor and Tabor, 1984). Polyamine deprivation also resulted in several ultrastructural abnormalities in yeast (Miret et al., 1992). Possibly, many of the roles that polyamines are thought to carry out, involving maintenance of basic cellular function (Tabor and Tabor, 1984), are common to all organisms. In addition, since their metabolism is affected by plant
hormones, and plants contain unusual biosynthetic pathways and conjugates, some functions of polyamines could be unique to plants. There is much that is not known about the normal role of polyamines in development that could be clarified by further investigation.
LIST OF REFERENCES


Bohner, J. and Bangerth, F. 1988. Cell number, cell size and hormone levels in semi-isogenic mutants of Lycopersicon pim pinellifolium differing in fruit size. Physiol. Plant. 72:316-320


Burtin, D., Martin-Tanguy, J., and Tepfer, J. 1991. $\alpha$-DL-Difluoromethylornithine, a specific, irreversible inhibitor of putrescine biosynthesis, induces a phenotype in tobacco similar to that ascribed to the root-inducing, left-hand transferred DNA of Agrobacterium rhizogenes. Plant Physiol. 95:461-468


Cheour, F., Willemot, C., Arul, J., Desjardins, Y., Makhlouf, J., Charest, P.M.,


Kyriakidis, D.A. 1983. Effect of plant growth hormones and polyamines on ornithine decarboxylase activity during the germination of barley seeds. Physiol. Plant. 57, 499-504


Lis, E.K. and Antoszewski, R. 1982. Do growth substances regulate the phloem as well as the xylem transport of nutrients to the strawberry receptacle? Planta 156:492-495


Mizrahi, Y., and Heimer, Y.M. 1982. Increased activity of ornithine decarboxylase in tomato ovaries induced by auxin. Physiol. Plant. 54:367-368


Thompson, P.A. 1967. Promotion of strawberry fruit development by treatment with growth regulating substances. Hort Res. 7:13-23


APPENDIX A

Data Relative to Chapter I
Fig. 7. Separation of benzoyl-polyamines by reverse-phase HPLC. A. Polyamine standards or B. Receptacle extracts (1 d after removal of achenes and application of α-NAA) were derivatized and separated as described in Chapter I, with water and methanol as solvents. Peaks: 1. PUT; 2, SPD; 3, diaminooctane (DAO); and 4: SPM.
Method for dansylation and TLC of polyamines

Perchloric acid extracts of strawberry receptacles were prepared as described previously (Chapter 1). The procedure for dansylation was modified from Flores and Galston (1982). An aliquot of the perchloric acid extract (200 μl) was combined with 400 μl of dansyl chloride (5 mg/ml in acetone, prepared fresh; Aldrich, Milwaukee, WI); and 200 μl saturated sodium carbonate in a 19 x 45 mm screw-cap vial. Vials were sealed with teflon-lined caps, vortexed for 10 s, and placed in a shaking water bath at 60 °C for 1 h. Excess dansyl reagent was removed by adding 100 μl of proline (100 mg/ml) and incubating for 30 min. Toluene (0.5 ml) was added and the contents vortexed for 30 s. The organic phase was collected and stored at -20 °C until subsequent analysis. Authentic polyamine standards were also dansylated as described above.

Prior to chromatography, the organic phase was dried at 50 °C under nitrogen, and the residue dissolved in an appropriate amount of toluene. In some cases involving separation of radiolabelled polyamines, the amount of radioactivity loaded on to each lane was increased by combining organic fractions derived from the same perchloric acid extract. For separation of labelled products, samples were fortified by addition of unlabelled standards that had been derivatized as described above. Chromatography was carried out by loading up to 50 μl of the toluene extracts on the pre-adsorbent zone of silica gel TLC plates (Whatman LK6D), and developing in chloroform:triethylamine (25:2; v/v). Unknowns were identified by comparison of \( R_f \) values with those of standards, following visualization under UV light. For determination of radioactivity, bands were scraped from the plate, mixed with scintillation cocktail (Hionic Fluor,
Packard Instrument Company, Meriden, CT) and subjected to liquid scintillation counting.
Fig. 8. Effect of removal of achenes and auxin application on strawberry receptacles. 'Allstar' fruits were de-achedened 10 d after pollination and treated with a lanolin paste lacking auxin (center), or with a lanolin paste containing α-NAA at 1 mM (right). Untreated fruits (left) were neither de-achedened nor treated with lanolin. Fruits/receptacles were harvested approximately 25 d after pollination.
Table 8. Effect of IAA on polyamine levels of strawberry receptacles. Untreated ‘Allstar’ receptacles were neither de-achened nor treated with lanolin; control receptacles were de-achened approximately 10 d after pollination and treated with a lanolin paste lacking auxin. IAA (1 mM) was applied to the surface of de-achened receptacles in a lanolin paste.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (after treatment)</th>
<th>Polyamine concentration</th>
<th>Polyamine content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PUT</td>
<td>SPD</td>
</tr>
<tr>
<td>Experiment I</td>
<td>d</td>
<td>nmol/g fresh weight</td>
<td>nmol/receptacle</td>
</tr>
<tr>
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<td>28±2</td>
</tr>
<tr>
<td>IAA</td>
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Experiment II

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</thead>
<tbody>
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<td></td>
<td></td>
<td>PUT</td>
<td>SPD</td>
</tr>
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<td>Untreated</td>
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<td>IAA</td>
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<td>62±5</td>
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*Values represent mean ±standard error for n=3 receptacles*
Table 9. Effect of polyamine biosynthetic inhibitors on growth of strawberry receptacles de-achened approximately 10 d after pollination. Control receptacles (cultivar Allstar) were treated with lanolin paste lacking auxin or inhibitors; DFMO, DFMA, and PUT were each at 2 mM, and α-NAA was at 1 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>Change in receptacle dimensions</th>
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</thead>
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<td></td>
<td>1 d after treatment</td>
<td>5 d after treatment</td>
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<tr>
<td>Control</td>
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<td>1.24±0.1</td>
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<tr>
<td>α-NAA</td>
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<td>2.17±0.2</td>
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<tr>
<td>α-NAA; DFMO; DFMA</td>
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<td>2.44±0.3</td>
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<tr>
<td>α-NAA; DFMO; DFMA; PUT</td>
<td>1.35±0.1</td>
<td>2.60±0.2</td>
</tr>
</tbody>
</table>

*Values represent the mean ± SE for n≥11 fruits or receptacles; †Change in fruit/receptacle dimensions 5 d after treatment
Fig. 9. Effect of polyamine biosynthetic inhibitors on total polyamine (PUT + SPD + SPM) content of strawberry receptacles. Receptacles (cultivar Allstar) were de-achened approximately 10 d after pollination and harvested 5 d later for polyamine analysis. Control receptacles were treated with lanolin paste lacking auxin or inhibitors; \( \alpha \)-NAA (NAA); \( \alpha \)-NAA + DFMO + DFMA (NAA + I); \( \alpha \)-NAA + DFMO + DFMA + PUT (NAA + I + PUT). DFMO, DFMA, and PUT were each at 2 mM, and \( \alpha \)-NAA was at 1 mM.
Fig. 10. Effect of polyamine biosynthetic inhibitors on total polyamine (PUT+SPD+SPM) concentration of strawberry receptacles. Receptacles (cultivar Allstar) were de-acheden approximately 10 d after pollination and harvested 1 or 5 d later for polyamine analysis. Control receptacles were treated with lanolin paste lacking auxin or inhibitors; α-NAA (NAA); α-NAA+DFMO+DFMA (NAA+I); α-NAA+DFMO+DFMA+PUT (NAA+I+PUT). DFMO, DFMA, and PUT were each at 2 mM, and α-NAA was at 1 mM.
Table 10. Metabolism of arginine applied to the surface of strawberry receptacles. 'Allstar' fruits were de-achened approximately 10 d after pollination, and 20 µl of solution containing L-U-[¹⁴C]-arginine (0.5 µCi; 1.46 nmol) in 5 mM sodium phosphate buffer (pH 7) with 1% DMSO and 0.05% Tween 80 (v/v), was added to the surface of each receptacle.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Receptacle fresh weight (g)</th>
<th>Radioactivity in perchloric acid extract (dpm/g fresh weight)</th>
<th>Radioactivity of PUT in perchloric acid extract (dpm/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.39</td>
<td>123120&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15720&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1.89</td>
<td>91842</td>
<td>10496</td>
</tr>
</tbody>
</table>

<sup>a</sup>Receptacles were harvested 24 h after treatment, rinsed with distilled water and polyamines were extracted as described elsewhere (Chapter 1); <sup>b</sup>Free polyamines were benzoylated and separated by HPLC, and fractions corresponding to PUT, SPD, and SPM were collected. Trace amounts of radioactivity were found in SPD, but no label was detected in SPM.
Table 11. Metabolism of PUT applied to the surface of strawberry receptacles. 'Allstar' fruits were de-achened approximately 10 d after pollination, and 25 µl of solution containing 1,4-[14C]-PUT (0.125 µCi; 1.09 nmol) in 10 mM sodium phosphate buffer (pH 7) with 2% DMSO and 0.1% Tween 80 (v/v), was added to the surface of each receptacle.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Receptacle fresh weight</th>
<th>Radioactivity in perchloric acid extract</th>
<th>Radioactivity of polyamines in perchloric acid extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>dpm/g fresh weight</td>
<td>dpm/g fresh weight PUT SPD</td>
</tr>
<tr>
<td>1</td>
<td>0.44</td>
<td>352655(^a)</td>
<td>205232(^b) 25200</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>574654</td>
<td>314274 26789</td>
</tr>
</tbody>
</table>

\(^a\)Receptacles were harvested 24 h after treatment, rinsed with distilled water and polyamines were extracted as described elsewhere (Chapter 1); \(^b\)Free polyamines were dansylated and separated by TLC, and bands corresponding to PUT, SPD, and SPM were scraped. Label was not detected in SPM, and approximately 20% of the label in the acid extract remained in the aqueous phase following partitioning of dansyl-polyamines into toluene.
Method of application of labelled PUT to bracts or leaves

Primary 'Allstar' strawberry flowers were tagged on the day of pollination. Either 10 or 15 d after pollination, all fruits and flowers other than the primary flower were removed from the inflorescence. The upper surface of the largest bract (unifoliate, and attached to the inflorescence approximately 40 mm below the primary fruit) was wiped (2x) with a cotton swab dipped in 95% ethanol. After allowing the bract to dry, 10 or 20 \( \mu l \) of 1,4-[\( ^{14} \text{C} \)]-PUT (50 \( \mu \text{Ci/ml} \); 115 mCi/mmol; Amersham Corporation, Arlington Heights, IL) was applied to the surface of the bract. One day later, fruits were harvested, rinsed thoroughly in distilled water, and polyamines were extracted. Dansylated polyamines were separated by TLC and radioactivity determined by liquid scintillation counting.

In some experiments, labelled PUT was applied to the surface of leaves. For these experiments, the stock of labelled PUT (50 \( \mu \text{Ci/ml} \)) was diluted with an equal volume of water. The middle leaflet of each of 4 fully expanded leaves was wiped with ethanol as described above, and 1.25 \( \mu \text{Ci} \) (50 \( \mu l \)) of PUT was applied to each of the middle leaflets (i.e., 5 \( \mu \text{Ci} \) was applied to each plant). Fruits were harvested 24 h later and analysed as described above.
Table 12. Radioactivity in polyamines extracted from 'Allstar' strawberry fruits following application of labelled PUT to bracts. A small volume (10-20 μl) of 1,4-[\(^{14}\)C] PUT (50 μCi/ml; 115 mCi/mmol) was added to the surface of a single bract attached to the inflorescence approximately 40 mm below the primary fruit.

<table>
<thead>
<tr>
<th>Fruit fresh weight (g)</th>
<th>Radioactivity in perchloric acid extract (dpm/g fresh weight)</th>
<th>Radioactivity of polyamines in perchloric acid extract (dpm/g fresh weight)</th>
<th>Distance migrated by label (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUT</td>
<td>SPD</td>
<td>SPM</td>
<td></td>
</tr>
<tr>
<td>Experiment I (0.5 μCi/replicate; 10 d after pollination)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.69</td>
<td>27871(^*)</td>
<td>2642(^*) 4602 1715</td>
</tr>
<tr>
<td>2</td>
<td>0.98</td>
<td>18228</td>
<td>1745 3341 932</td>
</tr>
<tr>
<td>Experiment II (1 μCi/replicate; 15 d after pollination)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.70</td>
<td>15523</td>
<td>1148 2573 430</td>
</tr>
<tr>
<td>2</td>
<td>2.07</td>
<td>27346</td>
<td>1504 3842 973</td>
</tr>
</tbody>
</table>

\(^*\)Fruits were harvested 24 h after application of label to bract, rinsed with distilled water and polyamines were extracted as described elsewhere (Chapter I); \(^*\)Free polyamines were dansylated and separated by TLC, and bands corresponding to PUT, SPD, and SPM were scraped and radioactivity was determined; \(^*\)Refers to the minimum distance that the label would have had to migrate in order to enter the fruit.
Table 13. Radioactivity in polyamines extracted from 'Allstar' strawberry fruits following application of labelled PUT to leaves. Ten days after pollination of the primary fruit, 50 μl of 1,4-[14C] PUT (25 μCi/ml; 57.5 mCi/mmol) was added to the upper surface of the middle leaflet of each of four fully expanded leaves (i.e., 5 μCi/plant; 1.25 μCi/leaf)

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Fruit fresh weight</th>
<th>Radioactivity in perchloric acid extract</th>
<th>Radioactivity of polyamines in perchloric acid extract</th>
<th>Distance migrated by label*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>dpm/g fresh weight</td>
<td>dpm/g fresh weight PUT SPD SPM</td>
<td>mm</td>
</tr>
<tr>
<td>1</td>
<td>0.50</td>
<td>16128*</td>
<td>1440^  3146  678</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>0.47</td>
<td>6827</td>
<td>476  1658  404</td>
<td>155</td>
</tr>
</tbody>
</table>

*Fruits were harvested 24 h after application of label to leaves (approximately 10 d after pollination), rinsed with distilled water and polyamines were extracted as described elsewhere (Chapter I); *Free polyamines were dansylated and separated by TLC, and bands corresponding to PUT, SPD, and SPM were scraped and radioactivity was determined; *Refers to the minimum distance that the label would have had to migrate in order to enter the fruit.
Fig. 11. Radiochromatogram obtained following TLC of fruit extracts. 'Allstar' fruits were labelled by application of radioactive PUT to strawberry leaves and harvesting the fruit 24 h later (○); control plants/fruits were not labelled (△). Extraction of polyamines, dansylation and TLC were performed as described elsewhere (Appendix A). Peaks: 1, PUT; 2, SPD; 3, SPM.
Wick-feeding of strawberry receptacles with polyamine biosynthetic inhibitors

Method: Primary 'Allstar' strawberry flowers were hand pollinated and tagged. Fruits at the green stage of development (5-6 d after pollination) were used for wick-feeding experiments. A cotton thread was attached with paraffin wax to the bottom of a 1.5 ml plastic microcentrifuge tube, and a fine needle was used to draw the thread through the cap of the microcentrifuge tube into the receptacle surface via the calyx. The thread was passed longitudinally through the fruit until it emerged at the apex. The needle was removed by cutting the thread at the apex, and the microcentrifuge tube was attached to a wooden stake so that the length of the thread between the mouth of the tube and the point of entry into the fruit was approximately 1.5-2.0 cm. Test solution (1.5 ml) was added to each microcentrifuge tube and the caps were closed. Three days later, after 90-95% of the solution had disappeared from the microcentrifuge tubes, vials and threads were discarded.

Observation: Test solutions from the moist wicks came into contact with achenes at the fruit apex. When test solutions containing DFMO+DFMA came into contact with achenes at the apex, the achenes turned dark brown to black in color and growth was reduced (Table 14, Appendix A). Since the inhibitory effect of DFMO+DFMA was not overcome by including PUT with the inhibitors, this method was not pursued further.
Table 14. Effect of wick-feeding polyamine biosynthetic inhibitors into developing ‘Allstar’ strawberry fruits. Untreated fruits were not subjected to wick-feeding. All test solutions were buffered with sodium phosphate at 10 mM (pH 7); DFMO, DFMA, and PUT were at 5 mM each. Fruits were harvested approximately 25 d after pollination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (g)</th>
<th>Diameter (mm)</th>
<th>Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.50±1.2</td>
<td>29.9±1.2</td>
<td>34.4±1.8</td>
</tr>
<tr>
<td>Control (buffer-treated)</td>
<td>12.51±1.2</td>
<td>29.7±1.1</td>
<td>33.6±1.2</td>
</tr>
<tr>
<td>DFMO + DFMA</td>
<td>9.33±0.8</td>
<td>27.5±1.0</td>
<td>28.9±1.4</td>
</tr>
<tr>
<td>DFMO + DFMA + PUT</td>
<td>8.45±1.0</td>
<td>26.3±1.4</td>
<td>27.4±1.3</td>
</tr>
<tr>
<td>PUT</td>
<td>12.96±0.7</td>
<td>30.9±0.6</td>
<td>33.1±1.4</td>
</tr>
</tbody>
</table>

*Values represent the mean±SE for n=7 fruits.
Culture of strawberry fruits in vitro

Based on a method optimized for study of in vitro development of 'Pajaro' strawberry fruits (Perkins-Veazie and Huber, 1992), attempts were made to culture 'Allstar' fruits in vitro.

Method: Approximately 12 d after pollination, primary fruits (green stage) were excised from greenhouse-grown 'Allstar' plants. The explants were rinsed with distilled water and the peduncles were trimmed so that the distance between the cut end and calyx was 100 mm. The cut end of the peduncle was placed in a solution (pH 4) of 30 g/L sucrose and 200 mg/L hydroxyquinoline hemisulfate in an autoclaved shell vial (25 x 95 mm). For some treatments, polyamine biosynthetic inhibitors (DFMO; DFMA; cyclohexylamine) and polyamines were included in the solution. The mouth of the vial was covered with sterile cotton so that the fruit remained outside the vial. At 2-3 d intervals, the peduncle was re-cut to remove 1-2 mm at the base and promote uptake.

Observations: Slight growth was observed in 'Allstar' fruits over a 10 d period with diameter and length of control fruits increasing by 3.0 and 1.8 mm, respectively (Table 15, Appendix A). Growth was considerably less than that reported for 'Pajaro' fruits, which exhibited increases in diameter and length of 7.0 and 5.7 mm, respectively, when excised and cultured 12 d after anthesis (Perkins-Veazie and Huber, 1992). The limited growth of cultured 'Allstar' fruits made it difficult to judge the effects of the inhibitor treatments, which appeared to have no effect on growth. In addition, in vitro culture caused changes in the developmental pattern of 'Allstar' fruits, so that cultured receptacles changed in color from green to pink without going through a white stage.
The limited growth of cultured fruits reported here in comparison with the results of Perkins-Veazie and Huber (1992), is probably a result of differences in the cultivars studied. Unlike 'Pajaro' fruits, fruits of 'Douglas' did not develop when cultured in vitro (Perkins-Veazie and Huber, 1992). Similarly, 'Allstar' fruits appear to be less responsive to in vitro culture than fruits of 'Pajaro'.

Attempts to culture 'Allstar' fruits in sterile, half-strength MS (Murashige and Skoog, 1962) salts with 15 g/L sucrose and 100 mg/L hydroxyquinoline hemisulfate were also unsuccessful. This method resulted in microbial contamination despite surface sterilization of the peduncle. The lack of an adequate seal for the vial (since a cotton plug was used instead of a plastic cap) may have contributed to the observed contamination. Cutting the base of the peduncle every 2-3 d could also have increased the chance of contamination.
Table 15. In vitro culture of 'Allstar' strawberry fruits. Fruits at the green stage of development (12 d after pollination) were cultured in a solution of sucrose (30 g/L) and hydroxyquinoline hemisulfate (200 mg/L). Inhibitors and/or polyamines, if present, were each at 1 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔDiameter</th>
<th>ΔLength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no inhibitors)</td>
<td>3.0±0.5</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>DFMO; DFMA; Cyclohexylamine</td>
<td>2.2±0.4</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>DFMO; DFMA; Cyclohexylamine; PUT; SPD</td>
<td>2.2±0.4</td>
<td>2.0±0.5</td>
</tr>
<tr>
<td>PUT; SPD</td>
<td>2.4±0.7</td>
<td>1.0±0.3</td>
</tr>
</tbody>
</table>

*Values represent the mean±SE for n ≥ 5 fruits cultured in vitro for 10 d.
Table 16. Preliminary experiment to determine the effect of auxin on polyamine concentrations of de-achened 'Selva’ strawberry receptacles. Secondary fruits at the green stage of development were de-achened and treated with lanolin paste lacking auxin (control) or with lanolin paste containing α-NAA (1 mM), and polyamine levels were determined 1 d after treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PUT</th>
<th>SPD</th>
<th>SPM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g fresh weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>88*</td>
<td>25</td>
<td>21</td>
<td>134</td>
</tr>
<tr>
<td>α-NAA</td>
<td>120</td>
<td>39</td>
<td>27</td>
<td>186</td>
</tr>
</tbody>
</table>

*Values represent data obtained from n = 1 receptacle
Table 17. Preliminary experiments to determine the effect of polyamine application on de-achened 'Selva' receptacles. Receptacles were de-achened and treated with a lanolin paste lacking auxin or polyamines (control). Auxin (α-NAA) or polyamines (PUT; SPD) were also supplied in the form of lanolin pastes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Increase in receptacle diameter after removal of achenes&lt;sup&gt;a&lt;/sup&gt; (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong> (Receptacles de-achened approximately 9 d after pollination)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-NAA (1 mM)</td>
<td>19.8</td>
</tr>
<tr>
<td>PUT; SPD (10 mM each)</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Experiment II</strong> (Receptacles de-achened 7 d after pollination)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Receptacle diameters were measured immediately after de-achening and 17 d later;<sup>b</sup>Values represent the mean for n ≥ 2 receptacles.
Table 18. Effect of β-NAA on polyamine concentrations of ‘Allstar’ strawberry receptacles. Data (from Experiment I; Table 1) expressed on a per gram dry weight (lyophilized) basis. Control receptacles were de-achened approximately 10 d after pollination and treated with lanolin paste lacking auxin; auxin-treated receptacles were also de-achened 10 d after pollination and treated with lanolin paste containing auxin at 1 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (after treatment)</th>
<th>Polyamine concentration</th>
<th>nmol/g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PUT</td>
<td>SPD</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>870±105*</td>
<td>478±31</td>
</tr>
<tr>
<td>β-NAA</td>
<td>1</td>
<td>1568±152</td>
<td>591±60</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>549±152</td>
<td>481±38</td>
</tr>
<tr>
<td>β-NAA</td>
<td>5</td>
<td>682±27</td>
<td>547±38</td>
</tr>
<tr>
<td>α-NAA</td>
<td>5</td>
<td>871±99</td>
<td>777±53</td>
</tr>
</tbody>
</table>

*Values represent the mean±SE for n=3 receptacles
Decarboxylation of amino acids by 'Allstar' strawberry extracts

Green fruits (approximately 10 d after pollination) were homogenized (~0.5 g fresh weight/ml) in extraction buffer (100 mM sodium phosphate, pH 8, 0.1 mM Na₂-EDTA, 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate), and centrifuged (20000xg; 4 °C). The resulting supernatant was dialysed (Spectra/Por membrane, 6000-8000 mw cutoff; Spectrum Medical Industries, Los Angeles, CA) overnight against extraction buffer prior to assay. To determine the effect of pH on decarboxylation of ornithine or arginine, the extract was dialysed against a mixture of 10 mM sodium phosphate (pH 8), 0.1 mM Na₂-EDTA, 2 mM dithiothreitol, and 0.1 mM pyridoxal phosphate. After dialysis, 40 µl of the extract was assayed for decarboxylase activity in a reaction mixture consisting of 80 mM sodium phosphate buffer (at desired pH), 0.04 mM pyridoxal phosphate, 0.04 mM Na₂-EDTA, 0.8 mM dithiothreitol, 0.5 mM unlabelled substrate (ornithine/arginine), and radiolabelled substrate (0.125 μCi of L-1-[¹⁴C]ornithine or 0.25 μCi of D,L-1-[¹⁴C]arginine). In addition, for measurement of arginine decarboxylation, unlabelled ornithine (20 mM) was included to inhibit arginase activity. The reaction was carried out at 37 °C for 60 min in sealed vials. Vials were transferred to an ice bath, 0.1 ml of trichloroacetic acid (10% w/v) was added, and tubes were incubated for an additional 45 min at 37 °C. The ¹⁴CO₂ released was trapped on filter paper impregnated with 25 µl of methylbenzethonium hydroxide and counted as described previously (Ponappa et al., 1992). Radiolabelled L-1-[¹⁴C]ornithine (54 mCi/mol) and D,L-1-[¹⁴C]arginine (49.5 mCi/mol) were purchased from Amersham Corp., Arlington Heights, IL, and Research Products International, Mount Prospect, IL., respectively.
Fig. 12. Effect of pH on decarboxylation of [14C]labelled ornithine by fruit extracts of strawberry. Experimental details are described in Appendix A (Decarboxylation of amino acids by strawberry extracts).
Fig. 13. Effect of pH on decarboxylation of [14C]labelled arginine by fruit extracts of strawberry. Experimental details are described in Appendix A (Decarboxylation of amino acids by strawberry extracts).
APPENDIX B

Data Relative to Chapter II
Fig. 14. Titration of 10 mM SPD (○) and 50 mM sodium phosphate (△). Each solution (100 ml; pH 7) was titrated using a Brinkman Digital Buret against 0.1 N NaOH and the change in pH recorded. A second 100 ml aliquot of each solution was similarly titrated against 0.1 N HCl.