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RADICAL SCAVENGING PROPERTIES OF POLYAMINES

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Abstract—Di- and polyamines are effective scavengers of free radicals generated in a number of chemical and *in vitro* enzyme systems. Free radical production was quantified spectrophotometrically using nitroblue tetrazolium and cytochrome *c* or by electron spin resonance. Levels of superoxide radical formed either enzymatically with xanthine oxidase or chemically from riboflavin or pyrogallol were significantly inhibited by spermine, spermidine, putrescine and cadaverine at 10 and 50 mM. The more reactive hydroxyl radical generated by the Fenton reaction was also effectively scavenged by di- and polyamines. In addition, the production of superoxide radical by senescing microsomal membranes was inhibited by di- and polyamines, as was the superoxide-dependent conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. The efficacy of polyamine-scavenging appears to be correlated with the extent of amination suggesting the involvement of amino groups. It is also apparent that some of the physiological effects of polyamines, in particular their propensity to inhibit lipid peroxidation and retard senescence, may be attributable to their radical-scavenging capability.

INTRODUCTION

Polyamines appear to be ubiquitous in living cells and have been implicated in a variety of regulatory processes ranging from promotion of growth and cell division [1] to inhibition of ethylene production and senescence [2–4]. For example, protoplasts are thought to be stabilized through polyamine-mediated inhibition of senescence [5, 6]. Exogenous polyamines prevent chlorophyll breakdown during dark-induced senescence of detached leaves [2] and also inhibit the rise in RNase and protease activities normally associated with senescence of leaf discs [7]. 1,3-Diaminopropane, a naturally-occurring oxidation product of polyamines, also retards leaf senescence [8], and plants exposed to a variety of stresses, ranging from mineral deficiencies through SO_2 fumigation to osmotic shock, often show enhanced levels of endogenous putrescine [9–12]. Putrescine levels also rise when oat leaf segments are incubated in media of low pH, and this is accompanied by an increase in the activity of arginine decarboxylase, one of the enzymes involved in putrescine biosynthesis [13].

At physiological pH, polyamines are fully protonated and polycationic [14]. It has been suggested that they achieve many of their physiological effects by associating with negative charges on nucleic acids and phospholipids, thereby stabilizing the function of chromosomes and membranes [15–17]. In the case of membranes, this contention is supported by the observation that polyamines significantly decrease the leakage of betacyanin during aging of beet root discs *in vitro* [2]. However, in other instances leakage accompanying senescence is not ameliorated by exogenous polyamines [18]. Other biophysical studies have indicated that polyamines influence membrane organization by associating with cytoskeletal elements. For example, Ballas *et al.* [19] have reported that polyamines stabilize erythrocyte membranes rendering them more resistant to fragmentation, probably through an association with cytoskeletal proteins on the cytoplasmic surface of the membranes. Moreover, the lateral mobility of erythrocyte membrane proteins is decreased by polyamines [20]. This effect is only observed when polyamines associate with sites on the cytoplasmic surface of the membrane, presumably cytoskeletal proteins, and is not accompanied by changes in lipid microviscosity.

Thus, the precise mechanisms by which polyamines exert their regulatory effects are unresolved. Kitada *et al.* [21] have recently reported that spermine inhibits NADPH- and ascorbic acid-dependent lipid peroxidation in liver microsomes, and they attributed their observations to an ability of spermine to associate with phospholipid and stabilize membranes. Lipid peroxidation is an inherent feature of senescence, and any tendency of polyamines to mitigate peroxidative reactions should retard senescence. In the present study, we provide evidence indicating that polyamines are effective scavengers of free radicals.

RESULTS

The free radical scavenging capabilities of di- and polyamines were evaluated using a variety of chemical and *in vitro* enzyme systems that generate the superoxide ($O_2^{\cdot-}$) radical. One of these, the conversion of xanthine to uric acid by xanthine oxidase, generates $O_2^{\cdot-}$ in sufficient quantities to be detectable with either nitroblue tetrazolium or cytochrome *c*. Spermine, spermidine, putrescine and cadaverine each inhibited the levels of $O_2^{\cdot-}$ generated by xanthine oxidase that were detectable using either of these methods (Table 1). The inhibitory effects were concentration-dependent, and the extent of inhibition appears to be related to the number of amino groups on the scavengers. For example, spermidine and spermine, which have three and four amino groups respectively,

Table 1. Scavenging of enzymatically-generated superoxide

Di/polyamine		Percentage of control A	B
Spermine	10 mM	76.5 ± 0.4	87.3 ± 0.6
	50 mM	42.7 ± 0.9	45.1 ± 0.7
Spermidine	10 mM	79.6 ± 1.0	67.7 ± 2.3
	50 mM	57.4 ± 2.6	35.1 ± 0.9
Putrescine	10 mM	91.6 ± 0.3	87.2 ± 5.9
	50 mM	80.6 ± 2.7	45.6 ± 0.3
Cadaverine	10 mM	80.0 ± 2.4	80.4 ± 4.0
	50 mM	43.8 ± 1.0	56.9 ± 2.7

Superoxide was generated by the xanthine/xanthine oxidase reaction. A, Detection by nitroblue tetrazolium; $A_{560\,nm}$ = 0.19/min for the control. B, Detection by cytochrome c; $A_{550\,nm}$ = 0.021/min for the control. Standard errors of the means are indicated; n = 3.

were more effective scavengers of $O_2^{\cdot-}$ than the diamines, putrescine and cadaverine (Table 1).

To test the possibility that polyamines were simply inhibiting xanthine oxidase rather than scavenging $O_2^{\cdot-}$, the abilities of di- and polyamines to scavenge $O_2^{\cdot-}$ generated chemically with either riboflavin or pyrogallol were also examined. The photochemical reaction with riboflavin can be monitored using Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid), which reacts with $O_2^{\cdot-}$ to form a semiquinone that is detectable by electron spin resonance (ESR) spectroscopy. Previous studies have demonstrated that the amplitude of the Tiron ESR spectrum is proportional to the amount of $O_2^{\cdot-}$ generated bv riboflavin [22]. Pyrogallol in basic solution rapidly autooxidizes forming $O_2^{\cdot-}$ and a coloured end product that can be quantified spectrophotometrically at 420 nm [23]. Polyamines proved capable of scavenging $O_2^{\cdot-}$ generated by either of these chemical reactions, and again the polyamines spermidine and spermine were more effective than the diamines (Table 2).

The hydroxyl radical ($OH^{\cdot}$), a more reactive species of oxygen than $O_2^{\cdot-}$, was generated chemically using the Fenton reaction and quantified using the diagnostic spin trap 4-(*N*-methylpyridinium)*t*-butyl nitrone (4-MePyBN). When 4-MePyBN was added to the Fenton reaction mixture, an ESR spectrum was obtained (Fig. 1), which, on the basis of its hyperfine splitting constants, can be attributed to the hydroxyl spin adduct of 4-MePyBN [24]. The amplitude of the spectrum is proportional to the amount of hydroxyl radical formed and is reduced by 10% when 25 mM spermidine is added to the Fenton reaction mixture and by > 70% in the presence of 50 mM spermidine (Fig. 2).

Table 2. Scavenging of chemically-generated superoxide

Di/polyamine		Percentage of control A	B
Spermine	10 mM	13.2 ± 0.5	56.1 ± 1.0
	50 mM	12.3 ± 0.4	40.6 ± 0.1
Spermidine	10 mM	16.9 ± 0.3	62.3 ± 1.6
	50 mM	13.7 ± 0.4	52.3 ± 1.6
Putrescine	10 mM	39.0 ± 1.2	84.6 ± 1.4
	50 mM	27.6 ± 0.8	74.3 ± 0.5
Cadaverine	10 mM	45.5 ± 1.0	88.5 ± 2.4
	50 mM	33.5 ± 1.0	79.5 ± 1.7

Superoxide was generated by illumination of riboflavin (A) and with pyrogallol (B). Standard errors of the means are indicated; n = 3. For (A), the amplitude for the control spectrum was 186 mm; for (B), $A_{420\,nm}$ = 0.053/min for the control.

Further confirmation of the scavenging abilities of di- and polyamines was obtained by examining their effects on the production of $O_2^{\cdot-}$ by microsomal membranes from senescing bean cotyledons. Superoxide production by these membranes is known to be mediated by a membrane-associated lipoxygenase [25] and can be monitored using Tiron. The ESR spectrum of the semiquinone formed when $O_2^{\cdot-}$ reacts with Tiron was completely eliminated by heat denaturation of the membranes (Fig. 3A, B), confirming that the $O_2^{\cdot-}$ was enzymatically generated. The amplitude of the Tiron ESR spectrum was also sensitive to di- and polyamines (Fig. 4). Again, the polyamines proved to be more effective scavengers than the diamines, reducing the amplitude of the Tiron ESR spectrum by up to 10% at a concentration of 0.1 mM and

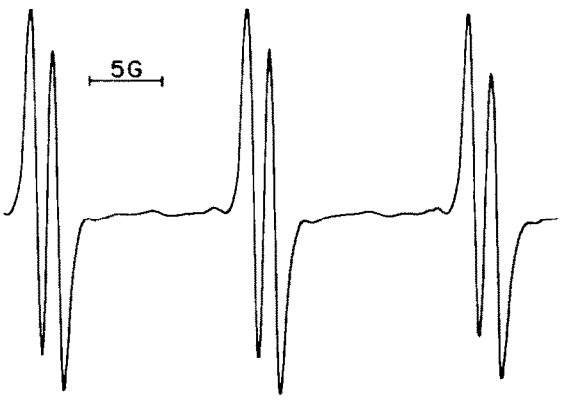

Fig. 1. ESR spectrum for the hydroxyl adduct of 4-MePyBN. Hydroxyl radicals were generated by the Fenton reaction.

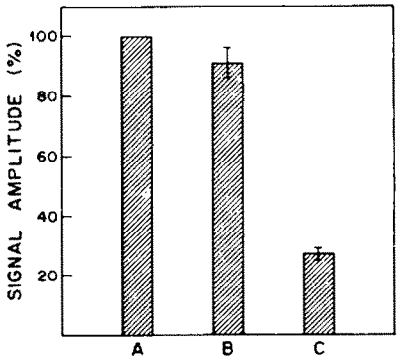

Fig. 2. Effect of spermidine on the amplitude of the second low field line of the spectrum for the hydroxyl adduct of 4-MePyBN. Hydroxyl radicals were generated by the Fenton reaction. A, Control; B, 25 mM spermidine; C, 50 mM spermidine. The amplitude for the control was 202 mm.

Fig. 3. Electron spin resonance spectrum for the Tiron semiquinone radical formed when Tiron reacts with superoxide. A, Tiron and smooth microsomes from 9-day-old bean cotyledons; B, Tiron and heat denatured microsomes.

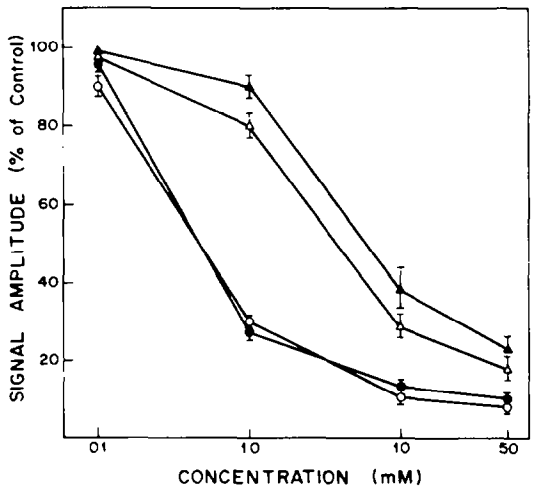

Fig. 4. Effects of polyamines on superoxide production by microsomal membranes from senescent bean cotyledons as measured by the amplitude of the Tiron radical ESR signal. ○, spermine; ●, spermidine; △, putrescine; ▲, cadaverine. Standard errors of the means are indicated when larger than the symbol; $n = 3$. The amplitude for the control was 99 mm.

by > 60% at 1 mM. By contrast, the diamines, putrescine and cadaverine, were virtually ineffective at 0.1 mM and had to be added at a concentration of 10 mM to cause 60% inhibition of the spectrum amplitude (Fig. 4).

The $O_2^{\cdot}$ produced by microsomal membranes has been shown to catalyse the conversion of ACC to ethylene [26], and the effects of di- and polyamines on this reaction were therefore also examined. The microsomal reaction differs from the native reaction by which ethylene is formed from ACC but is useful for studying the effects of scavengers in that it is mediated by lipoxygenase [27] and shows a strong dependence on superoxide [26]. The polyamines at 5–50 mM proved to be strong inhibitors of the conversion of ACC to ethylene by microsomes from pea epicotyls (Table 3). Putrescine and cadaverine (diamines) were also inhibitory, but to a smaller degree (Table 3).

Table 3. Effects of di and polyamines on the conversion of ACC to ethylene by microsomal membranes from pea epicotyls

Di/polyamine		Percentage of control
Spermine	5 mM	67.6 ± 9.7
	20 mM	42.1 ± 7.3
	50 mM	25.9 ± 2.9
Spermidine	5 mM	48.4 ± 7.4
	20 mM	57.3 ± 7.4
	50 mM	54.4 ± 11.2
Putrescine	5 mM	75.5 ± 10.0
	20 mM	54.2 ± 6.1
	50 mM	53.6 ± 3.1
Cadaverine	5 mM	109 ± 11.1
	20 mM	112 ± 7.9
	50 mM	95.2 ± 8.5

Standard errors of the means are indicated; $n = 3$. The control value was 1.43 nl ethylene/ml/hr.

DISCUSSION

There are several reports indicating that polyamines can delay senescence in plant tissue [2, 28] and that endogenous levels of polyamines decrease with advancing age in both plants and animals [2, 28, 29]. There is also evidence that age-related cell and organelle deterioration is due in part to free radical-mediated lipid peroxidation [30], and thus it is conceivable that the ability of polyamines to delay senescence reflects their propensity to scavenge activated oxygen. The changes in homeostasis that allow peroxidation to proceed in aging tissue are not understood, although in plant tissue both thylakoid and microsomal membranes have been shown to produce increased levels of superoxide with advancing senescence [22, 31]. Superoxide production by microsomes is attributable to a membrane-associated lipoxygenase that utilizes free fatty acids released from membrane phospholipid as substrate [25], and the enhanced production of $O_2^{\cdot}$ with advancing senescence induces alterations in the molecular organization of the lipid bilayer [31]. The concentrations of polyamines found to effectively scavenge the $O_2^{\cdot}$ produced by senescing microsomes in the present study are well within the concentration range (1–10 mM) that has been shown to delay senescence of plant tissues when applied exogenously [2]. Exogenous di- and polyamines (1–10 mM) have also been shown to inhibit the conversion of methionine and ACC to ethylene in petals of *Tradescantia*, discs and protoplasts of apple fruit and in leaf discs [4, 18]. These observations may also reflect the ability of polyamines to scavenge free radicals for the terminal step in this pathway is known to be sensitive to radical scavengers [32].

The ability of polyamines to decrease lipid peroxidation, delay senescence and inhibit ethylene production

has been attributed to their supposed ability to stabilize membranes by associating with negatively charged phospholipids [3, 4, 21, 33, 34]. This interpretation has been prompted, at least in part, by the observation that high concentrations of Ca^{2+}, which would compete with the polyamines for binding sites on the membrane phospholipids, prevent or partially reverse the polyamine effect [4]. However, Suttle [18] was able to distinguish between the effects of polyamines on ethylene biosynthesis and membrane stability in senescing petals of *Tradescantia*. Although ethylene production was inhibited by 60–70% in the presence of 1–10 mM polyamines, there was no commensurate inhibition of anthocyanin leakage reflecting membrane damage in the senescing petals. Thus, in *Tradescantia* the inhibition of ethylene production by polyamines is not accompanied by a reduction in membrane integrity as measured by leakage. It is therefore conceivable that the ameliorative effects of exogenous polyamines on senescence, lipid peroxidation and ethylene biosynthesis are all due to the scavenging of free radicals.

The role of endogenous polyamines in senescence is less clear. Levels of polyamines have been shown to decline with age for a number of senescing tissues [28]. However, in at least one system (senescing carnation petals), levels of putrescine rise during the early stages of senescence and decline again only after tissue deterioration is in an advanced state [35]. Thus the decline in polyamines does not correlate temporally with initiation of tissue deterioration.

EXPERIMENTAL

Free radical generation and detection. Superoxide generated enzymatically using xanthine oxidase was detected by measuring the reduction of nitroblue tetrazolium (NBT) [36] or cytochrome *c* [37]. For detection using NBT, the reaction mixture contained 0.05 mM xanthine, 0.025 mM NBT and 0.1 mM EDTA in 3 ml of 50 mM *N*-2-hydroxyethyl piperazine sulfonic acid (EPPS) buffer, pH 8. The reaction was initiated by adding 50 μl of xanthine oxidase soln (1.4 units/ml in the same buffer), and reduction of NBT at 25° was followed by measuring ΔA_{560} nm. For detection using cytochrome *c*, the reaction mixture contained 0.1 mM xanthine and 0.02 mM cytochrome *c* in 3 ml of 50 mM EPPS buffer, pH 8. The reaction was initiated by the addition of 30 μl of xanthine oxidase soln (1.7 units/ml in the same buffer), and reduction of cytochrome *c* at 25° was monitored at 550 nm. The effects of di- and polyamines on these reactions were determined by adding them directly to the reaction mixtures at specified concns. Superoxide radical generated photochemically by illumination of riboflavin was quantified by ESR using dihydroxybenzene disulphonic acid (Tiron) as described in ref. [22]. The reaction mixture contained 10 mM Tiron and 3 μg/ml riboflavin in 50 mM EPPS buffer, pH 8. Aliquots of the reaction mixture were taken up in 100-μl capillary tubes, which were inserted into a quartz sample holder in the microwave cavity of a Varian E-12 spectrometer. The reaction mixture was illuminated with an Hg-arc vapour lamp shone through 1 M $NaNO_3$ to remove UV radiation. Spectra were recorded at 25° 1 min later at a field setting of 3376 G, microwave frequency of 9.55 GHz, micropower of 10 mW, modulation amplitude of 2.0 G, time constant of 0.3 sec and scan time of 4 min.

Superoxide was also generated by autooxidation of pyrogallol [23] in a reaction mixture consisting of 1 mM EDTA in 3 ml of 50 mM EPPS buffer, pH 8. The reaction was initiated by adding 50 μl of 48 mM pyrogallol in 10 mM HCl and was followed at 25° by measuring the change in absorbance at 420 nm. The effects of di- and polyamines on this reaction were determined by adding them directly to the reaction mixture at specified concns.

Superoxide production by smooth microsomal membranes was measured by ESR using Tiron [31]. The membranes were isolated from senescent cotyledons of bean seedlings (*Phaseolus vulgaris* L. cv. Kinghorn) grown for 9 days in vermiculite at 29° in the dark [38]. The reaction mixture for measurement of superoxide production by the membranes consisted of 10 mM Tiron and 200 μg membrane protein/ml in 50 mM EPPS buffer, pH 8, and, when added, di- and polyamines at concens ranging from 0.1 to 50 mM. For expts with heat denatured microsomes, the microsomal suspension was immersed in a boiling water bath for 15 min. Reaction mixtures were incubated at 25° for 7 min and then were taken up in 100 μl capillary tubes, which were placed in a quartz sample holder in the microwave cavity of a Varian E-12 ESR spectrometer. Spectra were recorded at 25° 3 min later at a field setting of 3388 G, microwave frequency of 9.54 GHz, microwave power of 10 mW, modulation amplitude of 1.25 G, time constant of 1 sec and total scan time of 4 min. Protein was measured by the Bradford assay [39].

The hydroxyl radical was generated by the Fenton reaction and quantified by ESR using 4-(*N*-methylpyridinium)-*t*-butyl nitrone (4-MePyBN) as a diagnostic spin trap. The reaction mixture consisted of 50 μl of 1% H_2O_2 (v/v), 50 μl of 0.1 mM $FeSO_4 \cdot 7H_2O$, 50 μl of 200 mM 4-MePyBN, 2 mM EPPS, pH 8 and, when added, 25 or 50 mM spermine in a total vol. of 200 μl. All reagents were dissolved in double distilled water. Aliquots of the reaction mixture were immediately taken-up in 100-μl capillary tubes, which were inserted into a quartz sample holder in the microwave cavity of a Varian E-12 ESR spectrometer. After 2 min, spectra were recorded at 25° at a field setting of 3395 G, microwave frequency of 9.54 GHz, microwave power of 10 mW, modulation amplitude of 0.63 G, time constant of 0.3 sec and total scan time of 1 min.

Ethylene production. Microsomal membranes were isolated from epicotyl sections of 6-day-old pea seedlings (*Pisum sativum* L. cv. Alaska) grown in vermiculite in the dark at 29° [26]. The reaction mixture for measuring the O_2^--dependent conversion of ACC to ethylene by these membranes contained 150 μg membrane protein, 1 mM ACC, 50 mM EPPS buffer, pH 8 and, when added, 5, 20 or 50 mM di- and polyamines in a total vol. of 1 ml. Reaction mixtures were incubated at 31° for 1 hr in 12 × 100 mm test tubes, and ethylene was quantified by GC [26].

REFERENCES

1. Campbell, R. A., Bartos, D., Morris, D. R., Daves, G. D. Jr. and Bartos, F. (1978) *Advances in Polyamine Research*. Raven Press, New York.
2. Altman, A. (1982) *Physiol. Plant.* **54**, 189.
3. Ben-Arie, R., Lurie, S. and Mattoo, A. K. (1982) *Plant Sci. Letters* **24**, 239.
4. Apelbaum, A., Burgoon, A. C., Anderson, J. D. and Lieberman, M. (1981) *Plant Physiol.* **68**, 453.
5. Altman, A., Kaur-Sawhney, R. and Galston, A. W. (1977) *Plant Physiol.* **60**, 570.
6. Galston, A. W., Altman, A. and Kauri-Sawhney, R. (1978) *Plant. Sci. Letters* **11**, 69.
7. Popovic, R. B., Kyle, D. J., Cohen, A. S. and Zalik, S. (1979) *Plant Physiol.* **64**, 721.
8. Shih, L. M., Kaur-Sawhney, R., Fuhrer, J., Samanta, S. and Galston, A. W. (1982) *Plant Physiol.* **70**, 1592.
9. Coleman, R. G. and Richards, F. J. (1956) *Ann. Botany* **20**, 393.
10. Flores, H. and Galston, A. W. (1982) *Science* **217**, 1259.

11. Priebe, A., Klein, H. and Jager, H.-J. (1978) *J. Exp. Botany* **29**, 1045.
12. Smith, T. A. (1977) *Prog. Phytochem.* **4**, 27.
13. Young, N. D. and Galston, A. W. (1983) *Plant Physiol.* **71**, 767.
14. Morris, D. and Harada, J. J. (1980) in *Polyamines in Biomedical Research* (Gaugas, J. M., ed.) Wiley, New York.
15. Kaur-Sawhney, R., Altman, A. and Galston, A. W. (1978) *Plant Physiol.* **62**, 158.
16. Naik, B. I. and Srivastava, S. K. (1978) *Phytochemistry* **17**, 1885.
17. Cohen, A. S., Popovic, R. B. and Zalik, S. (1979) *Plant Physiol.* **64**, 717.
18. Suttle, J. C. (1981) *Phytochemistry* **20**, 1477.
19. Ballas, S. K., Mohandas, N., Marton, L. J. and Shohet, S. B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1942.
20. Schindler, M., Koppel, D. E. and Sheetz, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1457.
21. Kitada, M., Igarashi, K., Hirose, S. and Kitagawa, H. (1979) *Biochem. Biophys. Res. Commun.* **87**, 388.
22. McRae, D. G. and Thompson, J. E. (1983) *Planta* **158**, 185.
23. Marklund, S. and Marklund, G. (1974) *Eur. J. Biochem.* **47**, 469.
24. Janzen, E. G., Dudley, R. L. and Shetty, R. V. (1979) *J. Am. Chem. Soc.* **101**, 243.
25. Lynch, D. V. and Thompson, J. E. (1984) *FEBS Letters* **173**, 251.
26. McRae, D. G., Baker, J. E. and Thompson, J. E. (1982) *Plant Cell Physiol.* **23**, 375.
27. Lynch, D. V. and Thompson, J. E. (1985) *Planta* **164**, 121.
28. Altman, A. and Bachrach, U. (1981) in *Advances in Polyamine Research*, Vol. 3, pp. 365–375. Raven Press, New York.
29. Cohen, S. S. (1971) *Introduction to the Polyamines*. Prentice-Hall, Englewood Cliffs, NJ.
30. Harman, D. (1982) in *Free Radicals in Biology*, Vol. 5, pp. 255–275. Academic Press, New York.
31. Mayak, S., Legge, R. L. and Thompson, J. E. (1983) *Phytochemistry* **22**, 1375.
32. Apelbaum, A., Wang, S. Y., Burgoon, A. C., Baker, J. E. and Lieberman, M. (1981) *Plant Physiol.* **67**, 74.
33. Altman, A. (1982) *Physiol. Plant.* **54**, 194.
34. Altman, A., Friedman, R., Amir, D. and Levin, N. (1982) in *Plant Growth Substances*, pp. 483–494. Academic Press, New York.
35. Roberts, D. R., Walker, M. A., Thompson, J. E. and Dumbroff, E. B. (1984) *Plant Cell Physiol.* **25**, 315.
36. Beauchamp, C. and Fridovich, I. (1971) *Analyt. Biochem.* **44**, 276.
37. McCord, J. M. and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049.
38. McKersie, B. D. and Thompson, J. E. (1977) *Plant Physiol.* **59**, 803.
39. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.