The Effect of 6-Furfurylaminopurine on Senescence in Tobacco-Leaf Tissue after Harvest

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1. The concentration of chlorophyll decreased and the concentration of α -amino nitrogen and the absolute and specific activity of peptidase increased in disks of tobacco leaf incubated on water in darkness at 25°.
2. Although 6-furfurylaminopurine (kinetin) applied to the disks inhibited the increase in activity of peptidase after 4 days, this effect was too slow to account for the significant inhibition of increase in α -amino nitrogen after 24 hr.
3. Treating with kinetin also delayed loss of chlorophyll and soluble protein, though not until 4 and 8 days respectively.
4. The only significant effect of treating disks prepared from tobacco plants deficient in nitrogen with kinetin for 8 days was to inhibit the increase in α -amino nitrogen.
5. Adding kinetin (5 m μ M to 5 μ M) *in vitro* did not affect the activity of peptidase extracted from tobacco leaf.

6-Furfurylaminopurine (kinetin) applied to harvested leaves (Richmond & Lang, 1957) or to disks cut from leaves (Osborne, 1962; Sugiura, Umemura & Oota, 1962) delays loss of chlorophyll and protein. As treating with kinetin delays loss of RNA also (Osborne, 1962; Sugiura *et al.* 1962), the primary action of kinetin could be to maintain the mechanism of protein synthesis, a process involving several RNA fractions. However, inhibiting protein degradation by proteolytic enzymes also could be the primary action of kinetin. The experiments described here show that, although treating with kinetin inhibits the increase in specific activity of peptidase occurring in untreated leaf tissue after harvest (Anderson & Rowan, 1965), the effect is too slow to underlie the primary action of kinetin. The first effect observed was inhibition of the increase in concentration of α -amino nitrogen occurring in untreated tissue.

MATERIALS AND METHODS

The preparation of disks of tobacco leaf and the estimation of chlorophyll, α -amino N, soluble protein and activity of peptidase in the tissue were as described by Anderson & Rowan (1965). The chlorophyll in each sample was extracted in 25 ml. of ethanol (80%, v/v). Unless stated otherwise, disks were cut from leaves in the early stages of senescence (distinguished from mature leaves by lower chlorophyll content). After harvest, samples of leaf-disks (average mass per disk, 30 mg.) were floated abaxial side upwards on 10 ml. of glass-distilled water or solutions of kinetin in Petri dishes in darkness at 25°. The water or kinetin solutions were changed daily and did not become cloudy through infection with bacteria. Brown areas

appearing on disks treated with water were free of fungal infection when examined under the microscope. The least significant difference between two mean values was calculated by a *t* test after analysis of variance. Kinetin was supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

RESULTS

Effect of kinetin on changes in concentration of α -amino nitrogen and chlorophyll after harvest in disks cut from leaves of increasing physiological age. Three samples of leaf-disks were cut from each of a series of leaves from a mature plant. One sample from each leaf was analysed immediately for α -amino nitrogen and chlorophyll; the other two batches were treated with kinetin (50 μ M) or water respectively for 7 days. Treating disks cut from leaves of all ages with kinetin delayed degradation of chlorophyll and prevented the increase in α -amino nitrogen found in control disks (Fig. 1). The difference in concentration of chlorophyll in treated and control disks was greatest in disks cut from leaves nos. 10, 11 and 12. Since leaf no. 10 contained half the initial concentration of chlorophyll found in leaves of higher number, light-green leaves were used in subsequent experiments.

Effect of concentration of kinetin and duration of treatment on change in concentration of α -amino nitrogen, chlorophyll and soluble protein, and in activity of peptidase, in leaf tissue after harvest. Treating leaf-disks with kinetin at 1 μ M and above for 8 days prevented accumulation of α -amino nitrogen, and delayed the degradation of chlorophyll and soluble protein, and the increase in

absolute and specific activity of peptidase (Fig. 2). When samples of disks treated with $50\mu\text{M}$ -kinetin were analysed at regular intervals, the concentration of α -amino nitrogen decreased after 2 days but increased in control disks treated with water (Fig. 3); concentrations of $0.1\mu\text{M}$ and above inhibited the increase in α -amino nitrogen after 24 hr. (Fig. 4). However, treating with kinetin did not delay degradation of chlorophyll and soluble protein or the increase in activity of peptidase until day 4 or later (Fig. 3), and concentrations of $5\mu\text{M}$ and above caused no significant change in these fractions after 3 days.

Effect of nitrogen deficiency on change in concentration of chlorophyll and α -amino nitrogen, and in activity of peptidase, in leaf tissue after harvest. Plants were grown in soil and transferred to a complete liquid culture medium after 10 weeks. After a further 7 weeks, a matched pair of plants was transferred to a medium containing no nitrogen and a second matched pair was kept as a control in complete medium. Leaf-disks were cut from deficient and control plants 20 days after the transfer of the first pair of plants to deficient medium. Five samples per plant containing 20

disks each were prepared from leaves appearing to contain equal amounts of chlorophyll per unit area. One sample from each plant was analysed immediately and the remaining samples were analysed after treating either with water or with kinetin ($50\mu\text{M}$) for 4 and 8 days. In disks from plants cultured in nitrogen-deficient medium for 20 days, treating with kinetin did not significantly delay loss of chlorophyll or the increase in activity of peptidase found in control disks from nitrogen-sufficient plants (Fig. 5). However, treating with kinetin prevented increase in α -amino nitrogen in both sufficient and deficient disks after 4 days.

Changes in α -amino nitrogen in incubated disks examined by paper chromatography. The concentration of α -amino nitrogen in samples of leaf-disks on the day of harvest increased after incubating on water for 2 days, but decreased after incubating with $50\mu\text{M}$ -kinetin. Samples ($10\mu\text{l.}$) of the extracts in which α -amino nitrogen was determined were applied to Whatman no. 1 paper with appropriate marker spots of amino acids, and developed in butan-1-ol-acetic acid-water (4:1:1, by vol.).

Fig. 1. Effect of kinetin ($50\mu\text{M}$) on changes in concentration of α -amino N and chlorophyll after harvest in disks cut from leaves of increasing physiological age. After incubating for 7 days, the samples containing 16 disks were ground in 3.0 ml. of extracting medium. Day 0: $\circ$. Day 7: incubated on water, $\bullet$; incubated on kinetin, $\blacktriangle$.

Fig. 2. Effect of concentration of kinetin after incubating for 8 days on change in concentration of α -amino N ($\blacktriangle$) and chlorophyll ($\triangle$), and in absolute ($\bullet$) and specific activity ($\circ$) of peptidase. The arrow shows the concentration of α -amino N at harvest. Samples containing 26 disks were ground in 3.0 ml. of extracting medium. All results are means of duplicate extractions.

Fig. 3. Effect of kinetin on changes in concentration of α -amino N, chlorophyll and soluble protein and in absolute and specific activity of peptidase in leaf-disks with time after harvest. Samples of 25 disks were incubated on water (●) or $50 \mu\text{M}$ -kinetin (○) and ground in 3.0 ml. of extracting medium. Vertical lines show the least significant difference ($P < 0.05$) between two mean values. All results are means of duplicate extractions.

Replicate chromatograms run for 12 and 53 hr. were sprayed with ninhydrin or with the peptide spray of Rydon & Smith (1952). The predominant spots were glutamic acid, and peptides remaining at or close to the start line. Material with R_f equal to glutamic acid was eluted with water and the identification confirmed by chromatography in propan-2-ol-water (7:3, v/v). The intensity of all spots increased on chromatograms of extracts prepared after treating with water (Ranjan & Laloraya, 1960), but decreased after incubating on kinetin.

Effect of kinetin on the activity of peptidase in vitro. The activity of peptidase was measured in reaction mixtures containing kinetin between $5 \text{ m}\mu\text{M}$ and $5 \mu\text{M}$. Including kinetin in the reaction

Fig. 4. Effect of concentration of kinetin after incubating for 24 hr. on change in concentration of α -amino N. The arrow shows the concentration of α -amino N at harvest. Samples containing 10 disks were ground in 2.1 ml. of extracting medium. Results are means of duplicate extractions.

Fig. 5. Effect of kinetin on changes in concentration of α -amino N, chlorophyll and activity of peptidase in leaf-disks cut from tobacco plants grown with sufficient and deficient N. Samples containing 20 disks were incubated on water (with N, ●; without N, ▲) or $50 \mu\text{M}$ -kinetin (with N, ○; without N, △), and ground in 3.0 ml. of extracting medium. Results are means from duplicate plants.

mixtures did not change the activity of peptidase significantly.

DISCUSSION

In the experiments described here the most rapid response to treating leaf-disks of tobacco with kinetin was found in α -amino nitrogen. The effect on this fraction varied with the concentration of kinetin and the length of the treatment. In short experiments (24 hr.), $0.5 \mu\text{M}$ -kinetin prevented the increase in α -amino nitrogen found in the controls (Fig. 4), but concentrations of $1.0 \mu\text{M}$ and above caused a decrease in α -amino nitrogen. The effect was essentially the same in longer experiments (8 days), but the decrease in α -amino nitrogen at high concentrations of kinetin ($5.0 \mu\text{M}$ or more) was small compared with the increase in the absence of kinetin (Fig. 2). In both long and short experiments, concentrations of kinetin above $10 \mu\text{M}$ caused no further decrease in α -amino nitrogen.

Treating with kinetin did not delay significantly the increase in activity of peptidase and the decrease in concentration of chlorophyll and soluble protein until 4 days after harvest (Fig. 3). In disks from nitrogen-deficient plants, inhibition of the increase in α -amino nitrogen was the only significant effect of treating with kinetin for 8 days (Fig. 5). Since treating with kinetin inhibits the increase in α -amino nitrogen without preventing increase in peptidase activity, we conclude that kinetin delays protein degradation in harvested leaf tissue by stimulating protein synthesis rather than inhibiting degradation of protein by peptidase. Thus the decrease in α -amino nitrogen in disks treated with high concentrations of kinetin (Figs. 1–4) represents utilization in protein synthesis.

Since treating with kinetin inhibited the increase in α -amino nitrogen in harvested tissue, though it did not prevent soluble protein decreasing (Fig. 3), we conclude that the treatment induces synthesis of protein insoluble at pH 5.2. Decrease in glutamic acid and peptides observed on chromatograms of extracts prepared from tissue treated with kinetin suggests that these are major substrates for synthesis of this protein. Although protein of tobacco leaf contains 13–20% (w/w) of glutamic acid (Tso & McMurtrey, 1960), we cannot be certain if this amino acid is incorporated into protein directly, or donates α -amino nitrogen for synthesis of other amino acids.

The delay in increase of α -amino nitrogen caused by treating with kinetin for 24 hr. increases gradually between 0.05 and $10 \mu\text{M}$ (Fig. 4), and could provide a more rapid bioassay for kinins than the delay in degradation of chlorophyll proposed by Osborne & McCalla (1961).

Although adding kinetin *in vitro* has activated such hydrolytic enzymes as ribonuclease and deoxyribonuclease (Maciejewska-Potapczyk, 1959) and nucleotidase (Shuster & Gifford, 1962), this treatment did not affect the activity of the peptidase extracted from tobacco leaf.

Mothes and co-workers (Mothes, Engelbrecht & Kulajewa, 1959; Mothes, Engelbrecht & Schütte, 1961; Parthier, 1961) believed that amino acids accumulating in the area of a harvested leaf treated with kinetin (kinetin-directed transport) increased the rate of synthesis of protein in the area. In the experiments reported here, treating with kinetin delayed senescence in leaf-disks where kinetin-directed transport cannot take place. If the amount of amino acids in harvested leaves regulated the rate of synthesis of protein, decrease in concentration of protein would never occur.

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