Interaction of Naphthaleneacetic Acid and Kinetin in the Senescence of Detached Leaves

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Summary. Kinetin and naphthaleneacetic acid were applied separately and in combination to excised leaf disks, localized areas of laminae, and petioles of detached leaves of broccoli (Brassica oleracea L., var. italica) and Xanthium (Xanthium pensylvanicum Wallr.). Senescence (measured as loss of chlorophyll) was strongly retarded by kinetin, but very slightly influenced by naphthaleneacetic acid. When the 2 substances were applied concurrently, the effect of kinetin was markedly reduced by naphthaleneacetic acid. Neither interference with uptake nor transport of kinetin appeared to cause the reduction.

In broccoli, the response to kinetin was as great in young leaves as in old leaves, and the reduction of the kinetin effect by naphthaleneacetic acid was greater in young leaves than in old leaves. Results indicate that the prevention of loss of critical material may be more significantly related to the delay of senescence by kinetin than is directed transport and accumulation (mobilization). Kinetin may control 2 or more events which contribute independently to the delay of senescence in detached leaves, and naphthaleneacetic acid may possibly interfere with only 1 of them.

Phytohormones are known to delay senescence in detached leaves (16) and excised leaf tissue (15). Auxins retard senescence in detached leaves of some species in which phytohormones are ineffective (14, 17). Since both phytohormones and auxins have been shown to influence senescence, it is pertinent to investigate their interaction. It has been reported that auxins did not interfere with phytohormone bioassays based upon the retention of chlorophyll in leaf tissues of Xanthium (15) or of barley (2).

Retention of chlorophyll by benzyladenine (BA)-treated broccoli heads was not altered by auxins (1). It is shown in the present report that, although α-naphthaleneacetic acid (NAA) alone had very slight influence upon the rate at which chlorophyll content declined in detached leaves of broccoli and Xanthium, the chlorophyll-maintaining effect of kinetin was greatly reduced in the presence of NAA. The magnitude of this interaction was influenced in broccoli by the age (or ontogenetic position) of the leaf.

The mechanism by which phytohormones delay senescence in detached leaves is not known. Both the maintenance of RNA and protein syntheses (13, 18, 19) and the directed transport and accumulation (together called mobilization) of various substances (9, 10, 11) have been correlated with the control of senescence by phytohormones. The present report suggests that the prevention of loss of critical material may play a more significant role than mobilization in the delay of senescence by kinetin.

Materials and Methods

Plant Material. Xanthium pensylvanicum Wallr. (inbred strain from California Institute of Technology, courtesy of Professor James Bonner) was germinated and grown in a greenhouse, under 18-hour photoperiod, until selected plants were bearing from 12 to 15 fully expanded leaves. Leaves were then selected for uniformity of size, color, and position on the plant.

Broccoli (Brassica oleracea L., var. italica, cv. Coastal) was grown in the field. Leaves were selected for uniformity and according to an arbitrary age classification which was based upon ontogenetic position and color. The oldest leaf used, classified as pre-senescent, was the one immediately above the most distal leaf then showing the distinct yellow flush characteristic of the onset of senescence. Leaves classified as mature were those undergoing the subtle color change from dark blue-green to lighter green, but distally removed.

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from the pre-senescent leaf by a minimum of 2 intervening leaves. Leaves classified as young were the most distal which had achieved maximum or nearly maximum expansion, were deep blue-green in color, and distally removed from a nature leaf by a minimum of 3 intervening leaves.

Selected leaves of Xanthium and broccoli were severed at the base of the petiole, and the petiole was placed immediately in sterile, deionized water. Leaves were then transferred to a 20°C room, where they were kept (aged) until used. A light regime of 12 hours of dim, diffuse fluorescent light (ca. 90 ft-c mixed warm white and daylight) alternating with 12-hour dark periods was maintained continuously, except as noted for excised tissue disks.

Contamination Control. Since long incubation periods were characteristic of these experiments, the reduction and evaluation of microbial contamination were of primary concern. Standard sterile techniques were adapted. The petiole was swabbed with 1.5% (v/v) NaOCl before the leaf was severed from the plant, and the entire leaf was then washed in sterile deionized water. The base of the petiole was re-cut and all media were replaced at 24-hour intervals. Flasks in which the leaves were held were loosely plugged with cotton. No satisfactory decontamination treatment was found for leaf tissue. Penicillin G (280 units per ml) and dihydrostreptomycin sulfate (45 μg per ml) were not effective.

Extraction and Estimation of Chlorophyll. Tissue samples were extracted directly with boiling 80% ethanol. The extracts were cooled and diluted to a concentration suitable for spectrophotometry, and optical density was read at 665 mμ. In experiments with excised disks, chlorophyll was expressed in arbitrary units as the product of optical density and the final volume (in ml) of the extract, \( \times 10^2 \) divided by the initial weight (in mg) of the tissue. No correction was made for specific absorbance. If tissue weight was not determined before treatment, chlorophyll was expressed simply as optical density per standard volume of extract.

Experimental Solutions. Kinetin, NAA, and mixtures of the 2, were prepared in deionized water in a range of concentrations from 1 μM to 50 μM. All solutions were sterilized by autoclaving. Solutions used as media for the incubation of leaf disks of broccoli and for surface application to broccoli leaves included Tween-20 (0.005%, v/v).

Application of Active Compounds. Excised Tissue. To shorten the period of incubation after excision, detached young leaves of broccoli were aged from 8 to 10 days before use, and mature leaves were aged from 3 to 5 days, according to the rate of color change. Presenescent leaves were used without aging. A set of disks (8 mm in diam) was excised from the smallest leaf area which could provide 1 disk for each of a series of test solutions. Excisions were located to exclude major vascular tissue from the disks. Immediately following its excision, each tissue disk was placed in one of the test solutions, the order being randomized within each set of disks. Ten sets of disks were excised from 5 or more leaves, providing a total of 10 disks per test solution. Each sample of 10 disks was blotted, weighed, and placed in a petri dish with the abaxial surfaces of the disks contacting a filter-paper liner saturated with its test solution. Duplicate samples were then prepared in the same way. The disks were incubated in a water-saturated atmosphere at 20°C, in darkness except during observation and transfer. After 3 to 4 days, when controls incubated on water were visibly yellowing, each sample of the series was extracted for estimation of the chlorophyll content.

Xanthium leaves were aged for 3 days. Tissue disks were then prepared and treated in the same manner as for broccoli.

Localized Surface Application. Freshly detached broccoli leaves were bisected from the apex through the base of the petiole, providing 2 comparable half-leaves. The distal third of the midvein was too small to divide evenly and was excised. The petioles were rinsed, and each lateral half-leaf was placed in an individual flask with the petiole in water. At the midpoint of each half-leaf, a plastic cylinder 2 cm in diameter and 8 mm high was attached to the adaxial leaf surface by a film of lanolin paste. A disk of thick filter paper was placed in each cylinder. The paper disk on 1 of each matched pair of half-leaves was saturated with 0.4 ml of water or other control solution; the disk on the other half-leaf received the test solution. After 2 hours the paper disks were removed. This application was repeated to provide three 2-hour treatments during each 12-hour light period for 3 successive days. Loss of chlorophyll was visually estimated as the percent of maximum yellowing. When the more senescent treated area of a pair appeared to have lost about 80% of its chlorophyll, tissue samples were taken as follows: From each treated 20-mm circle a disk 15 mm in diameter was excised, extracted, and the extract made to 10 ml. From the untreated area, ten 8-mm disks were excised, extracted, and the extract diluted to 28 ml. This dilution schedule allowed a direct comparison of the optical densities of the extracts on the basis of surface area of the tissue.

Local application of substances to the adaxial surface of Xanthium leaves was carried out in the same manner, but the leaves were not bisected.

Application by Petiole Uptake. Freshly detached broccoli leaves were bisected as previously described. The petiole of 1 of each pair of half-leaves was placed in 50 ml of control solution, and the petiole of the other half-leaf was placed in test solution. When controls were estimated to be approximately 80% yellowed, twenty 8-mm disks were excised in random pattern from the control half-leaf, and the sampling pattern was geometrically duplicated in the other half-leaf of the pair. The 2 samples were extracted for estimation of chlorophyll.
Fig. 1. Chlorophyll content of excised leaf disks after incubation in the dark on the indicated solutions, expressed in arbitrary units (OD × volume of extract in ml × 10⁻³ + initial wt of tissue in mg) and calculated as the average value from extracts of 2 sets of 10 disks. *, control; ○, kinetin alone; ●, kinetin (5 µM) + varied concentrations of NAA; □, NAA (15 µM) + varied concentrations of kinetin; ■, NAA alone. A) Young broccoli leaves aged 9 days, disks incubated 4 days. B) Mature broccoli leaves aged 5 days, disks incubated 4 days. C) Pre-senescent leaves not aged, disks incubated 3 days. D) Xanthium leaves aged 3 days, disks incubated 3 days.
Results

Excised Tissue. The loss of chlorophyll in excised disks of young broccoli leaves was retarded by kinetin (fig 1A). The response to NAA alone was slight and variable and was probably not significant. However, when the tissue disks were incubated in the presence of both kinetin and NAA, the chlorophyll-maintaining effect of kinetin was markedly depressed. The maximum concentration (50 μM) of NAA used in these experiments did not nullify the effect of an intermediate concentration of kinetin, nor did the maximum concentration (50 μM) of kinetin completely overcome the opposing effect of NAA. The results of comparable experiments with mature and pre-senescent leaves are shown in figures 1B and 1C. Tissue samples excised from leaves of the 3 ages were very similar in their responses to kinetin or NAA separately, but differed in response to the 2 substances in combination. The reduction of the kinetin effect by NAA was weaker in tissue of mature leaves than in that from young leaves and did not occur, or was slightly reversed, in tissue from pre-senescent leaves.

Excluding the effect of leaf age, which was not investigated, the response of leaf disks of Xanthiun (fig 1D) closely resembled that of broccoli.

Localized Surface Application. The data given in table I are from a representative experiment, in which 10 bisected broccoli leaves were used for comparison of each pair of treatments. The response of the sharply defined treated area to kinetin and to NAA resembled closely the response of excised disks. The rate of chlorophyll decline was strongly retarded by kinetin, but was only slightly and variably influenced by NAA. When kinetin and NAA were applied in combination, the kinetin effect was greatly reduced in young leaves and somewhat less reduced in mature leaves. These data show clearly that the untreated area of a kinetin-treated half-leaf retained a higher chlorophyll content than either the treated or untreated part of the water-treated control half-leaf. Excellent agreement was obtained between half-leaves of control pairs.

In similar experiments, the response of whole detached Xanthiun leaves was much like that of broccoli leaves. NAA applied concurrently with kinetin markedly reduced the chlorophyll-maintaining effect of kinetin. The untreated area of the kinetin-treated side of a leaf lost no more chlorophyll than the water-treated control side.

Application by Petiole Uptake. The effect of kinetin upon chlorophyll retention in the lamina of bisected broccoli leaves appeared to be similar, whether the regulatory substance was applied to the petiole or directly to the leaf surface. NAA alone had little effect and, as in the preceding experiments, NAA applied concurrently with kinetin markedly reduced the measured effect of kinetin. The depression of the kinetin effect by NAA was greater in young apical leaves than in older basal leaves of broccoli.

Discussion

The methods used to apply kinetin and NAA to leaf tissues have provided 3 distinct experimental situations, and the information so obtained is applicable to examination of the following subjects.

Correlation Between Mobilization and Senescence. Associated with the retardation of senescence in kinetin-treated areas of detached leaves, directed transport and accumulation (mobilization) of metabolites are induced by the kinetin locus. Mothes et al. (9) and Muller and Leopold (10) have concluded, furthermore, that senescence is accelerated in those areas of the leaf which are exterior to the kinetin locus. Leopold and Kawase (4) have reported control by BA of the senescence of leaflets on a segment of bean stem: senescence.

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<th>Table I. Effect of Localized Surface Application of Kinetin and N.A.A on Chlorophyll Content of Bisected Broccoli Leaves</th>
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<td>Mean OD (665 μm) of extracts from 10 half-leaves. Paired treatments were applied to pairs of half-leaves. Concentrations of kinetin and NAA were 20 μM each. Direct comparisons may be made among all 4 values for 1 pair of 1 age. If the superscripts following compared values are unlike, the difference is significant (p = 0.05 or less).</td>
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<th>Treatment</th>
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<td>Duration (days)</td>
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(loss of chlorophyll) was retarded in a treated leaf, but induced in an untreated leaf elsewhere on the stem. This was interpreted as mobilization of nutrients by the BA-treated leaf and consequent deprivation of the untreated leaf.

Despite this evidence, there are aspects of senescence which are not explained convincingly by the mobilization concept (3, 5). Mobilization cannot be implicated in the ability of kinetin to delay senescence in excised disks of leaf tissue (fig 1). Osborne (13) examined the development of senescence in Xanthium leaf tissue excised in the shape of 2 disks connected by a bridge of tissue. When 1 of the disks was treated with kinetin, the presence of the connected untreated disk did not augment the effect of kinetin in the treated disk, and the untreated disk did not senesce more rapidly in the presence of the kinetin-treated tissue. Thus, whether or not mobilization occurred, it did not influence the rate of senescence in either disk. We have found that localized application of kinetin to 1 half-leaf of a bisected broccoli leaf (table 1) resulted in a small but significant retention of chlorophyll by the untreated area of the kinetin-treated half-leaf, as compared to any part of the control half-leaf. The influence of kinetin was not completely restricted to the treated area, although the demarkation remained sharp. One possible explanation of this unexpected observation is that the control half-leaf may have suffered a loss of substance from the lamina into the petiole, while corresponding loss was prevented in the kinetin-treated half-leaf. An analogous prevention of loss or of efflux may be suggested as a possible explanation of the effect of kinetin in excised leaf disks. The strong retention of chlorophyll resulting from application of kinetin to the petiole of a broccoli leaf was quite uniform throughout the lamina. As in the case of excised disks, it is unlikely that mobilization played any part in the delay of senescence, since this method of application did not establish a kinetin locus. Moreover, there was no source from which mobilization might occur other than the petiole itself.

For each of the 3 methods of application, then, it may be argued that 1 senescence-correlated role of kinetin may be described better as a prevention of efflux than as an induction of mobilization. The critical aspect of such efflux might well relate to a specific substance. For example, Oota and Takata (12) have suggested that the senescence of plant organs is associated with the outward movement of RNA.

Interaction Between Kinetin and NAA. In each of the 3 methods of application, the ability of kinetin to delay the loss of chlorophyll was depressed by NAA. The interaction between kinetin and NAA was not caused by interference with kinetin transport, since the NAA effect was fully expressed in disks treated after excision. Interference with kinetin uptake was not the cause, since the interaction occurred even when application of kinetin to the petiole was terminated prior to application of NAA.

The concentration-response curves for excised disks of broccoli leaves (fig 1A, 1B) show that NAA at concentrations as high as 50 μM did not nullify the effect of a moderate concentration of kinetin (5 μM). The shape of the curve indicates, indeed, that the saturation concentration for NAA was quite low and that greater NAA concentration probably would be no more effective. Conversely, increased concentration of kinetin did not appear to nullify the countereffect of NAA (15 μM). These relationships seem anomalous, particularly since the expression of the auxin effect was dependent upon exogenous kinetin. It is possible that kinetin may control 2 or more critical events which contribute independently to the delay of senescence in detached leaves. There is strong evidence in the literature to indicate such a dichotomy, although it is not certain that the 2 effects of phyto kinins are independent. Mothes and his associates (6, 7, 8, 9, 20) have concluded that directed transport and accumulation of amino acids and other substances by a kinetin locus is the primary function of kinetin, and that RNA and protein syntheses are a consequence of the accumulation. Osborne (13) and Sugiiura et al. (19) have presented the contrasting opinion that the primary effect of kinetin upon leaf senescence is the maintenance of RNA synthesis, and hence protein synthesis. One of the most critical problems in phyto kinin physiology concerns the orientation of these 2 roles. We pose the possibility that auxin may interfere with 1 of the functions but not the other.

Influence of Leaf Age or Ontogenetic Position. The data relating the age of broccoli leaves to the magnitude of response show 2 surprising aspects. First, the response of young leaves to exogenous kinetin was as great or greater than the response of old leaves. This is in contrast to the results of Mothes (6) and Mothes and Engelbrecht (7), who reported that kinetin induced the accumulation of labeled glycine only slightly in young leaves of Nicotiana, but strongly in old leaves. They speculated that the young leaf contained an adequate supply of a native phyto kinin and, therefore, did not respond to exogenous kinetin. Similarly, Osborne and Hallaway (14) have shown that the effectiveness of an auxin in maintaining protein levels in detached leaves of Prunus was greater in old leaves than in young leaves.

The second noteworthy effect of leaf age was the decreasing magnitude of the depression of NAA of the kinetin effect with increasing age of the leaf. In near-senescent leaves the auxin either had no effect or slightly increased the ability of kinetin to delay the loss of chlorophyll. This would not appear to relate to a gradient of endogenous auxin, since the NAA saturation concentration (fig 1A, 1B)
was no greater for disks of mature leaves than
for disks of young leaves.

We have not satisfactorily separated the influence of leaf age from the influence of ontogenetic position. If young broccoli leaves were detached and then aged for 8 days before treatment with kinetin and NAA, the auxin was still able to depress the chlorophyll-maintaining effect of kinetin: the leaf still responded as a young leaf in this respect. Either the process of senescence in detached leaves differs from the process in attached leaves, or the response gradient reported here is determined by something other than age.

**Literature Cited**


