Phosphorus-induced Leaf Abscission in Detached Shoots of Olive and Citrus

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ABSTRACT. Previous studies have demonstrated that phosphorus, which stimulates ethylene biosynthesis, induces abscission of olive leaves directly without the involvement of ethylene. In the present study this possibility was further explored by comparing the effects of an ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG), and an ethylene action inhibitor, 2,5-norbornadiene (NBD), in olive [*Olea europaea* (L.) 'Manzanillo'] and citrus [*Citrus sinensis* (L.) Osbeck 'Shamouti']. In olive, leaf abscission was always induced in the presence of KH_2PO_4 with or without AVG and NBD (alone or in combination), but it was much more pronounced when KH_2PO_4 was applied alone. In citrus, KH_2PO_4 did not induce leaf abscission in the presence of NBD during the first 48 (detached shoots) or 60 hours (leaf explants) despite the high levels of ethylene production by the tissues. Our results demonstrate that phosphorus can, at least partly, act independently of ethylene action in inducing leaf abscission in olive but not in citrus.

Recent observations that citrate-phosphate buffer induces leaf abscission in olive leaf explants (Banno et al., 1993) has led to new strategies for the worldwide goal of obtaining olive fruit abscission in the management of olive fruit harvesting. Elimination experiments revealed that the abscission-inducing factor was phosphorus. Banno et al. (1993) reported that phosphorus (in the form of NaH_2PO_4) also induced leaf and fruit abscission in stem-fed olive explants, and that foliar sprays with the same compound caused fruit abscission with minimal leaf loss. They further reported that ethylene evolution in the various shoot tissues in which NaH_2PO_4 accumulated was quite low. These data, as well as the suggestion that phosphorus inhibits ethylene biosynthesis in some higher plants (Fuchs et al., 1981; Sobolewska and Plich, 1986), prompted investigations to determine whether phosphorus causes abscission directly or indirectly via ethylene production.

The potential contribution of ethylene to NaH_2PO_4 -induced abscission in olive was investigated by Burnik-Tiefengraber et al. (1994) using aminooxyacetic acid (AOA), an inhibitor of ethylene biosynthesis (Amrhein and Wenker, 1979). It was found that AOA did not measurably affect leaf abscission or ethylene evolution induced by NaH_2PO_4 . Moreover, the observed NaH_2PO_4 -induced increase in ethylene evolution fell far short of the exogenous concentrations of ethylene needed to induce olive leaf abscission, and leaf abscission induced by NaH_2PO_4 occurred more rapidly than that induced by ethylene treatment. Since the response and sensitivity to a particular inhibitor of ethylene biosynthesis may vary between tissues (Yang, 1980; Yang and Hoffman, 1984), Yamada and Martin (1994) compared AOA, aminoethoxyvinylglycine (AVG), and CoCl_2 , all inhibitors of ethylene biosynthesis, to establish whether the observed NaH_2PO_4 -induced leaf abscission in olive was mediated through elevated ethylene evolution. Of the three inhibitors, only AVG inhibited ethylene production from detached olive shoots that were stem-fed with NaH_2PO_4 .

Surprisingly, all inhibitors promoted leaf abscission when administered alone or with NaH_2PO_4 . Of special interest was the finding that AVG almost completely inhibited ethylene production but promoted leaf abscission in the presence and absence of NaH_2PO_4 , suggesting that AVG by itself may have a direct effect on leaf abscission. Based on their studies, Yamada and Martin (1994) suggested that NaH_2PO_4 -induced leaf abscission in olive may occur independently of endogenous ethylene evolution.

Since Martin's group used inhibitors of ethylene biosynthesis, which usually failed to block ethylene biosynthesis completely (Yamada and Martin, 1994), it was impossible to eliminate the possible involvement of even small quantities of ethylene in the NaH_2PO_4 -induced leaf abscission in olive. The purpose of the present study was to extend the study on phosphorus-induced leaf abscission in detached olive shoots using 2,5-norbornadiene (NBD) as an inhibitor of ethylene action (Sisler et al., 1985). KH_2PO_4 was used instead of NaH_2PO_4 to prevent possible phytotoxic effects of high Na^+ (Stassart et al. 1981 and Ben-Hayyim et al. 1987). Citrus leaf abscission was used as a reference. Our results demonstrate that KH_2PO_4 can, at least partly, act independently of ethylene action in inducing leaf abscission in olive but not in citrus.

Materials and Methods

PLANT MATERIAL. The effect of KH_2PO_4 on ethylene production and abscission was studied in 1-year-old detached vegetative shoots (8 to 10 cm long) sampled from 15-year-old olive (*Olea europaea* L. 'Manzanillo') trees and in 1-year-old detached shoots (16 to 18 cm long) sampled from 18-year-old orange (*Citrus sinensis* [L.] Osbeck 'Shamouti') trees grown in sandy-loam soil at the experimental farm of the Faculty of Agriculture at Rehovot. Olive shoots with six to seven pairs of leaves, and citrus shoots with eight to ten leaves were sampled in the morning and immediately transferred to the laboratory in plastic bags. In one experiment citrus leaf explants, each consisting of 10 mm of leaf blade and 10 mm of petiole (Ratner et al., 1969), were used. For each treatment, two leaf explants were placed in a 1.5-mL Eppendorf vial containing 1 mL of treatment solution (10 replications per treatment).

KH_2PO_4 , AVG, AND NBD TREATMENTS. Eight vegetative detached

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Fig. 1. Effect of NBD ($4000 \mu\text{L}\cdot\text{L}^{-1}$) on ethylene production (A and B) and leaf abscission (C and D) induced by stem feeding with KH_2PO_4 at different concentrations (50 or 75 mM) in 'Manzanillo' olive detached shoots. NBD was applied at zero time. Each detached shoot was 8 to 10 cm long with six to seven pairs of leaves. Vertical bars indicate means $\pm$ SE ($n = 8$).

olive shoots per treatment were pretreated by immersion for 5 min in distilled water or in AVG solution at a concentration of 0.01, 0.1, 1, or 10 mM. Each shoot was wiped, its basal end was recut under water, and the whole shoot was placed in an open 30-mL vial containing 2 mL of treatment solution. In some experiments, the above treatment cycle was repeated every 24 h for up to 4 d after daily recording of the rates of abscission and ethylene production as described below. In all experiments with olive, the treatment solutions also contained $200 \text{ mg}\cdot\text{L}^{-1}$ 8-hydroxyquinoline citrate (8-HQC) as a fungistat to prolong shoot life >4 d, since in the control treatments abscission reached only 40% (Yamada and Martin, 1994). The solutions were adjusted to pH 5.0 with either diluted KOH or HCl solutions.

Eight vegetative detached citrus shoots per treatment were pretreated with AVG as described above for olive shoots, and the cut basal end of the stem was recut in water and placed in a 5-mL vial containing 3 mL of treatment solution. It was not necessary to add 8-HQC to the treatment solution, since experiments with citrus were terminated not later than 72 h, when abscission in the control reached $\approx 90\%$.

For NBD treatment, vials containing either detached olive or citrus shoots were placed in 26-L air-tight plastic containers. Before the containers were closed, NBD (Aldrich Chemical Co., Milwaukee, Wis.) solution ($480 \mu\text{L}$ calculated to give $4000 \mu\text{L}\cdot\text{L}^{-1}$ NBD after evaporation) absorbed onto Whatman no. 1 filter paper (8×3 cm) was introduced. In each container, two plastic beakers containing 2×4 cm Whatman no. 1 filter paper and 2 mL of 40% KOH served as CO_2 traps. The containers were kept in a temperature-controlled growth room (22 to 25°C). Each day the containers were opened, the shoots were removed, and the empty containers were aerated for 10 min. Fresh NBD and CO_2 traps were introduced every day.

ETHYLENE MEASUREMENT. Every 24 h, test tubes containing the olive shoots, with their base immersed in 2 mL treatment solution, were closed with serum caps for 1 h. Each citrus shoot, with its base immersed in 5 mL of treatment solution, was sealed in an airtight plastic bag equipped with a tube closed with a serum cap. Immediately after evacuation of the air, 100 mL of fresh air were injected into the bag. For ethylene measurements of citrus leaf explants, each pair of citrus leaf explants was transferred with the 1.5-mL Eppendorf vial into a 10-mL test tube containing 1 mL of treatment solution to ensure continuous uptake, and the test tubes were closed with serum caps for 1 h. After 1 h of incubation, 1-mL air samples were taken for ethylene measurement and injected into a Varian 3300 Analytical Gas Chromatograph equipped with an alumina column at 100°C and a flame ionization detector at 120°C . In the case of NBD treatment, NBD was ventilated from the open vials before they were closed for ethylene measurement.

ABSCISSION MEASUREMENT. Leaf abscission of citrus (Ratner et al., 1969) and olive (Lang and Martin, 1989) was examined every 24 h.

STATISTICAL ANALYSIS OF DATA. Each experiment was repeated at least twice with eight replications per treatment. Ethylene data are expressed on a per-leaf basis as described earlier (Goren, 1993; Ratner et al., 1969; Yamada and Martin, 1994) disregarding the presence of the shoots since their ethylene production relative to leaves was negligible. All data were tested by analysis of variance.

Results

EXPERIMENTS WITH OLIVE IN THE PRESENCE OF NBD AND AVG. Exposure of detached olive shoots to KH_2PO_4 alone (50 or 75 mM) for 7 d yielded results (Fig. 1 A and C) similar to those of Yamada and Martin (1994), who used 75 mM NaH_2PO_4 for 10 d. The effects of KH_2PO_4 on ethylene production and leaf abscission in the

Fig. 2. Effect of AVG (0.1 to 10 mM), with (B and D) or without (A and C) NBD ($4000 \mu\text{L}\cdot\text{L}^{-1}$), on ethylene production (A and B) and leaf abscission (C and D) induced by stem feeding with KH_2PO_4 (50 mM) in 'Manzanillo' olive detached shoots. For further details, see legend to Fig. 1.

Fig. 3. Effect of preincubation with AVG (0.1 mM) and NBD (4000 $\mu\text{L}\cdot\text{L}^{-1}$), on ethylene production (A) and leaf abscission (B) induced by stem feeding with KH_2PO_4 (50 mM) in 'Manzanillo' olive detached shoots. KH_2PO_4 was applied 0 h (C), 3 h (B) or 24 h (D) after AVG and NBD treatment. For further details, see legend to Fig. 1.

presence of NBD was further studied. Based on preliminary experiments, 4000 $\mu\text{L}\cdot\text{L}^{-1}$ NBD was selected for the present experiments since at higher concentration, some phytotoxic effects were observed in the presence of KH_2PO_4 . In spite of the fact that this treatment stimulated ethylene production (compare Fig. 1B with Fig. 1A), abscission was reduced compared to KH_2PO_4 alone (compare Fig. 1D with Fig. 1C). Since some phytotoxic effects were observed with 75 mM KH_2PO_4 , all further experiments were conducted with 50 mM KH_2PO_4 .

In further experiments, AVG was included to inhibit the increased ethylene production, which was induced by the combination of KH_2PO_4 and NBD. Application of AVG alone did not induce ethylene production (Fig. 2A). Combination of KH_2PO_4 with up to 10 mM AVG and NBD, induced ethylene production after 2 d of incubation (Fig. 2B). The lowest AVG concentration (0.1 mM) was unable to reduce the KH_2PO_4 - and NBD-induced ethylene production (Fig. 2B). Application of AVG alone (1 or 10 mM) stimulated leaf abscission (Fig. 2C), and the addition of KH_2PO_4 accelerated leaf abscission (Fig. 2D). Although the course of leaf abscission was slowed down in the presence of NBD and AVG at the lower concentration (0.1 mM), by day 6 the rate of leaf abscission still reached 80% (Fig. 2D).

As shown above (Fig. 2B), NBD and AVG in the presence of KH_2PO_4 induced ethylene production after a lag period of 2 d (Fig. 3A). Ethylene production reached a peak after 4 d and decreased later on. When KH_2PO_4 was added 3 or 24 h following pretreatment with AVG (0.1 mM) and NBD, almost no ethylene production was observed for 3 d after the beginning of the experiment. A small increase in ethylene production was recorded after 4 d (Fig. 3A). Delaying KH_2PO_4 treatment by 3 or 24 h caused a delay in leaf abscission (Fig. 3B).

An attempt was made to increase the efficiency of the uptake of AVG by subjecting

detached olive shoots up to four treatment cycles over 4 d (see Material and Methods). It was assumed that this procedure would increase the inhibitory effect of the low AVG concentration (0.1 mM) on KH_2PO_4 -induced abscission. After three or four treatment cycles, the inhibitory effect of AVG on ethylene biosynthesis was enhanced (Fig. 4A), but 60% leaf abscission was nevertheless recorded (Fig. 4C). In the presence of NBD, three and four cycles of AVG treatment yielded the strongest inhibition of ethylene biosynthesis (Fig. 4B), yet two cycles of AVG treatments caused 40% inhibition of abscission after 7 d, when the experiment was terminated (Fig. 4D).

EXPERIMENTS WITH CITRUS IN THE PRESENCE OF NBD AND AVG. KH_2PO_4 (25, 50, or 75 mM) induced ethylene production in detached citrus shoots after 18 h (Fig. 5A). The maximum effect of KH_2PO_4 on ethylene production in citrus shoots was obtained between 24 and 36 h after the initiation of the experiment, with no significant differences between the three KH_2PO_4 concentrations up to 48 h. Ethylene production in the controls (without added KH_2PO_4) was significantly lower than that in the treated shoots; it started only 24 h after the beginning of the experiment and reached a maximum at 36 h (Fig. 5A). Leaf abscission in control shoots began between 24 and 36 h after the initiation of the experiment (Fig. 5B). Treatment with KH_2PO_4 induced leaf abscission at all concentrations (Fig. 5B). The strongest effect was obtained with the highest concentration (75 mM), which induced 100% abscission after 48 h. At the same time, 20% abscission was obtained in the control without KH_2PO_4 , 55% abscission with 25 mM, and 75% with 50 mM (Fig. 5B). In all subsequent experiments with citrus, 50 mM KH_2PO_4 was used to avoid the phytotoxic effects observed with 75 mM KH_2PO_4 .

Yamada and Martin (1994) showed that AVG inhibited NaH_2PO_4 -induced ethylene evolution in olive shoots but that NaH_2PO_4 was still able to induce leaf abscission in the presence of AVG. In detached citrus shoots, AVG (0.025, 0.05, or 0.1 mM)

Fig. 4. Effects of repeated immersion in AVG (0.1 mM), with (B and D) or without (A and C) NBD (4000 $\mu\text{L}\cdot\text{L}^{-1}$), on ethylene production (A and B) and leaf abscission (C and D) by stem feeding with KH_2PO_4 (50 mM) in 'Manzanillo' olive detached shoots. Shoots were immersed, wiped, recut at the basal end, and placed in treatment solutions every 24 h (one cycle) for up to 4 d. For further details, see legend to Fig. 1.

Fig. 5. Effect of stem feeding with different concentrations of KH_2PO_4 on ethylene production (A) and leaf abscission (B) in 'Shamouti' orange detached shoots. Each detached shoot was 16 to 18 cm long with 8 to 10 leaves. Vertical bars indicate means $\pm$ SE ($n = 8$).

reduced KH_2PO_4 -induced ethylene formation (Fig. 6A) and KH_2PO_4 -induced leaf abscission (Fig. 6B), as recorded 72 h after the initiation of the experiment.

Similar to previous reports (Sisler et al., 1985), NBD applied at $4000 \mu\text{L}\cdot\text{L}^{-1}$ to citrus shoots, induced ethylene production between 48 and 72 h after the initiation of the experiment (Fig. 7A), but blocked its action, as indicated by the lack of abscission up to 48 h (Fig. 7B). Addition of KH_2PO_4 increased ethylene production (Fig. 7A) and resulted in $\approx 40\%$ abscission after 72 h (Fig. 7B). At this stage, untreated control citrus shoots had already reached 80% abscission. However, when KH_2PO_4 was applied alone, 100% abscission was observed at this period (Fig. 7B).

In leaf explants, the effects of AVG and NBD in the presence of KH_2PO_4 were similar to those obtained with detached shoots but much more pronounced (Fig. 8). No leaf abscission occurred until 60 h in the presence of NBD (Fig. 8B) even at the highest KH_2PO_4 concentration and in spite of high ethylene production (Fig. 8A), which was induced by NBD.

Discussion

The findings of this study on KH_2PO_4 -induced leaf abscission in olive shoots are in general in agreement with those of Banno et al. (1993), Burnik-Tiefengraber et al. (1994) and Yamada and Martin (1994).

On the basis of their study, using the ethylene biosynthesis inhibitor AVG (2.5 and 5 mM), Yamada and Martin (1994) proposed that KH_2PO_4 , which stimulates ethylene biosynthesis, induces abscission in olive detached shoots independent of ethylene. However, the observed occurrence of abscission in the present study (Fig. 2C) in the absence of ethylene (Fig. 2A), when using the above mentioned concentrations of AVG, even without KH_2PO_4 , led us to suspect that leaf abscission might at least partly, be the result of AVG-induced toxicity. Lower AVG concentrations (0.1 mM) also efficiently blocked ethylene biosynthesis (Fig. 2A) with no abscission (Fig. 2C). The addition of KH_2PO_4 to AVG (0.01 and 0.1 mM) resulted in some ethylene production which was accom-

panied by a significant increase in abscission, although less than the control (data not presented). Thus, even low levels of endogenous ethylene in the abscission zone might be enough to stimulate the abscission process, although usually much higher concentrations of exogenously ethylene are applied (Goren et al., 1988; Lang and Martin, 1987; 1989; Yamada and Martin, 1994). It should also be noted that, at the abscission zone, ethylene sensitivity and reactivity most probably vary with the nature and age of the tissue, as well as with the season (Goren, 1993).

Using the citrus leaf explant system, Sisler et al. (1985) demonstrated that NBD acts as a competitive inhibitor of ethylene-induced abscission. In olive, NBD, even at relatively high concentrations (up to $4000 \mu\text{L}\cdot\text{L}^{-1}$) and in the presence of KH_2PO_4 (50 and 75 mM) caused $\approx 50\%$ to 60% abscission (Fig. 1D). NBD and KH_2PO_4 induced ethylene production (Figs. 1 A and B). Under these conditions, NBD inhibited abscission only by 40% (Fig. 1D). Unfortunately, due to the phytotoxic symptoms, which appeared when using higher NBD concentration, it was impossible to raise the NBD concentration, as previously done in citrus (Sisler et al., 1985) in order to try to completely compete with the ethylene effect on abscission.

In an attempt to answer the question whether it is possible to separate between ethylene and KH_2PO_4 -induced abscission, AVG (0.1 mM) was applied together with KH_2PO_4 . Some ethylene was always observed in the presence of KH_2PO_4 or NBD alone or together in the presence of AVG (Fig. 2B). Both inhibitors had only modest effects on the inhibition of KH_2PO_4 -induced leaf abscission (Fig. 2D).

To improve the effect of AVG on the inhibition of ethylene biosynthesis, olive shoots were immersed in AVG solution attempting to increase AVG uptake above that was achieved by basal stem feeding. This is especially important when a low AVG concentration is used (Goren and Bukovac, 1973). This application technique indeed reduced much ($\approx 90\%$) the induction of ethylene by NBD and KH_2PO_4 (Fig. 4B). KH_2PO_4 -induced abscission was also reduced (Fig. 4D) but relatively much less than ethylene production ($\approx 60\%$). Multiple AVG treatments and con-

Fig. 6. Effect of different concentrations of AVG (up to 0.1 mM) on ethylene production (A) and leaf abscission (B) induced by stem feeding with KH_2PO_4 (50 mM) in 'Shamouti' orange detached shoots as recorded 72 h after the initiation of the experiment. For further details, see legend to Fig. 5.

Fig. 7. Effects of NBD (4000 $\mu\text{L}\cdot\text{L}^{-1}$) on ethylene production (A) and leaf abscission (B) induced by stem feeding with KH_2PO_4 (50 mM) in 'Shamouti' orange detached shoots. NBD was applied at zero time. For further details, see legend to Fig. 5.

Fig. 8. Effects of NBD (4000 $\mu\text{L}\cdot\text{L}^{-1}$) on ethylene production (A) and leaf abscission (B) induced by petiole feeding with KH_2PO_4 (25 or 50 mM) in 'Shamouti' orange leaf explants. Each leaf explant consisted of 10 mm of leaf blade, the abscission zone, and 10 mm of the petiole. NBD was applied at zero time. Vertical bars indicate means $\pm$ SE ($n = 10$).

tinuous presence of NBD delayed, but did not prevent completely, the onset of KH_2PO_4 -induced leaf abscission.

Contrary to the results obtained with olives, KH_2PO_4 was unable to induce leaf abscission in the presence of NBD, alone or together with ethylene (Figs. 7A and 8A), up to 48 h with detached shoots (Fig. 7B) and up to 60 h with leaf explants (Fig. 8B).

The evidence indicating that the mechanism of KH_2PO_4 -induced citrus leaf abscission is distinct from that of P-induced ethylene production is of great interest and calls for further study. It should, however, be noted that for all combinations of feeding treatments, abscission was inhibited by no more than 40% when AVG concentrations were low. We can, nevertheless, conclude that KH_2PO_4 is capable of inducing olive leaf abscission directly, since there was at least 40% abscission in the combined presence of AVG and NBD. It should, however, be emphasized that this phenomenon is not common to all plants, since the KH_2PO_4 -induced leaf abscission in citrus was shown here to be caused by ethylene.

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