ABSORPTION AND UTILIZATION OF FOLIAR-APPLIED NITROGEN IN PRUNUS SPP.

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David Ronald Leece

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ABSTRACT

ABSORPTION AND UTILIZATION
OF FOLIAR-APPLIED NITROGEN
IN PRUNUS SPP.

By

David Ronald Leece

Foliar sprays containing nitrate-nitrogen have recently come into use in commercial stone fruit production. The present investigations evaluated nitrate foliar sprays as a nitrogen source for Prunus spp. and studied their absorption with a view to improving their efficiency.

Although stone fruit trees normally metabolise nitrate in their roots it was postulated that the leaves would also be able to metabolise nitrate and hence be in a position to benefit from nitrate foliar sprays if absorbed. Nitrate reductase, the first enzyme in the metabolic sequence from nitrate to amino acids, was used to indicate nitrate metabolism, and, to avoid the complication of cuticular penetration, nitrate was supplied to the leaf via the petiole.

A 15mM nitrate solution was applied to the roots of young, sand-cultured apricot, sour cherry, sweet cherry,

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peach and plum trees. Nitrate was subsequently found in the leaves of all species and an NADH-dependent nitrate reductase was extracted from the leaves of all species except peach. The apricot enzyme was two to three times as active as the enzymes from the other species including that from an apple control. It was shown to be substrate (10mM NO_3^-) inducible and able to use either NADH or $FMNH_2$ as its electron donor. Thus it would seem to be typical of nitrate reductase as commonly found in plant leaves.

The enzyme extraction procedure was based on the use of insoluble polyvinylpyrrolidone (PVP.) in the extraction medium to remove phenolic compounds which would otherwise have inactivated the enzyme. When nitrate reductase was extracted from oat in the presence of each of the stone fruit tissues, PVP. provided adequate protection in the presence of apricot and plum tissues, but only partial protection with sour cherry and sweet cherry, and no protection with peach tissue. Hydrolyzable tannins not removed by PVP. were believed responsible for this inactivation. Incorporation in the extraction medium of reducing agents and inhibitors of o-diphenoloxidase *viz*. cysteine, mercaptobenzothiazole, and dithiothreitol, at concentrations up to 10mM, gave no additional protection to the oat enzyme. Thus the failure to find nitrate reductase in peach tissue was ascribed to enzyme inactivation during extraction by hydrolyzable tannins.

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Nitrate reductase was detected in leaves of field-grown apricot, sour cherry and plum trees. This indicated that field-grown leaves of Prunus spp. could metabolise nitrate and that they should be able to utilize nitrate foliar sprays, provided the nitrate ions could enter the leaves through the cuticle.

Sand-culture evaluations of nitrate foliar sprays showed that K^+ was likely to be the most effective carrier ion for nitrate foliar sprays when applied to stone fruit trees and that both the carrier ion and the nitrate were absorbed.

Field evaluations of potassium nitrate foliar sprays were conducted on nitrogen deficient, young, commercial peach trees for two seasons. Neither three autumn (post harvest) nor three spring applications of KNO_3 or a urea control at 0.2 to 0.4 g. equiv. N per litre raised leaf nitrogen significantly nor corrected tree nitrogen deficiency. However a soil nitrogen application (0.5 lb. N per tree post harvest) raised leaf nitrogen concentration, increased terminal growth and trunk circumference, and corrected the nitrogen deficiency. It was concluded that insufficient nitrogen or potassium were absorbed from the foliar sprays to be able to influence tree nitrogen or potassium status or growth. This was attributed to lack of cuticular penetration by KNO_3 and urea. Working with excised leaf discs it was found that partial removal of

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epicuticular wax doubled cuticular penetration by 0.4 M KNO_3. It was concluded that the commercial practice of using nitrate sprays at 0.07 g. equiv. per litre would have no influence on the nitrogen status or growth of stone fruit trees.

ABSORPTION AND UTILIZATION OF FOLIAR-APPLIED NITROGEN IN *PRUNUS* SPP.

By

David Ronald Leece

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INTRODUCTION

Nitrogen is the principal nutrient required by fruit trees, which, in commercial practice, has to be supplied as a fertilizer (Johnston and Larson 1965). As such it constitutes an important cost factor in fruit production and thus warrants investigations leading to its more efficient use. Such investigations have received impetus recently from the rising public concern over fertilizer contamination of water supplies, particularly by nitrate ions which can be toxic to both animals (Lewis 1951) and humans (Altman and Dittmer 1968).

Commercially, nitrogen is normally supplied to fruit trees as a soil fertilizer application. Up to one pound of elemental nitrogen is given to each mature tree in autumn or winter (Johnston and Larson 1965) to build up root and rootstock storage reserves which the tree relies on for spring growth (Taylor 1967). A supplemental application of up to half a pound may be applied in spring to assist fruit development (Keatly et al. 1968). Soil applications of nitrogen are subject to losses due to leaching (principally as nitrate ions), soil fixation, denitrification, and to bad placement in relation to tree roots. These losses, particularly those due to leaching, have

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contributed to the build up of nitrate ions in the ground water (Tisdale and Nelson 1966) and subsequently in agricultural and domestic water supplies.

One approach to improving the efficiency of nitrogen fertilizer applications has been to partially or completely replace soil applications with foliar applications of nitrogen in the form of urea sprays. This has proved successful for apple trees (Fisher 1952) and Citrus spp. (Jones and Embleton 1965) but not for stone fruit (Prunus spp.) or pear trees (Benson 1953). Foliar applications of urea in spring to apple trees have permitted more efficient regulation of tree nitrogen supplies, than have corresponding soil applications. As a result problems of insufficient nitrogen for fruit development or of excess nitrogen as the fruit approaches maturity have been more easily avoided (Fisher 1952). Other advantages of these foliar sprays have been that trees low in vigor due to low temperature (winter) injury or root damage have responded more rapidly to them than to soil applications (Benson 1953), and the losses associated with soil applications have been avoided.

However, there are some disadvantages associated with foliar sprays. The natural way for fruit trees to absorb nutrients is via their roots not their leaves. Leaf absorption is sometimes minimal, particularly as the leaf ages (Wittwer and Teubner 1959). Losses may occur during application through excess spray drifting to

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adjacent plantings or dripping to the ground (it may subsequently be absorbed by feeder roots which are concentrated at the periphery of trees - an example of good soil fertilizer placement), and rainfall soon after application may wash or leach spray material from the leaves (again these losses would be well placed for root absorption).

Recently commercial interests have marketed nitrogen foliar sprays containing urea and nitrate-nitrogen (principally as potassium nitrate), and these are now being used in commercial fruit production in Michigan, South Africa (Bester et al. 1965) and other fruit growing areas. Growers and industry representatives have claimed that potassium nitrate sprays aid recovery of sweet cherry, peach, and plum trees from potassium deficiency and winter injury (Kenworthy 1965). It is well established that urea has little value as a nitrogen foliar spray for Prunus spp., but no field evaluations of nitrate sprays have been reported. The investigations now reported were carried out to evaluate nitrate foliar sprays as a nitrogen source for Prunus spp. and to study their absorption with a view to improving their efficiency.

REVIEW OF LITERATURE

Nitrogenous fertilizer was first applied to fruit trees in the form of a foliage spray when Hamilton et al. (1943) sprayed apple, cherry and peach trees in the spring with solutions of urea, sodium and potassium nitrate, and ammonium sulphate at concentrations from 0.6 to 1.2 per cent.[1] The only favorable response obtained was an increase in apple tree nitrogen resulting from the urea sprays. A great many field evaluations of nitrogen foliar sprays for all commercially important fruit tree species rapidly followed. It soon became apparent that urea was the best form of nitrogen for foliar sprays, to which apple and citrus trees responded very favorably, while stone fruit trees failed to show any response.

In this review the nitrogen nutrition of fruit trees will first be examined to see at what points in the annual rhythm of root absorption, storage and mobilization of nitrogen, foliar nitrogen sprays might prove beneficial.

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[1] In this thesis, in all reports of field evaluations of foliar sprays, concentration has been expressed on a per cent basis. Appendix A gives a more complete explanation.

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Next the results of the many field and greenhouse evaluations of nitrogen foliar sprays will be studied and finally the possible reasons for the anomalous behavior of stone fruit trees will be considered.

Possible Roles for Nitrogen Foliar Sprays in the Nitrogen Nutrition of Deciduous Fruit Trees

Taylor (1967a) has recently reviewed the annual rhythm of storage and mobilization of nitrogen in deciduous fruit trees. The cycle commences with the creation of nitrogen storage reserves in woody tissues during autumn and winter. The nitrogen absorbed by the fine roots at this time is stored principally in the larger roots or rootstock wood and from 20 to 80 per cent of the nitrogen in the leaves migrates to woody storage tissue, mainly the bark of scion wood, in autumn prior to leaf fall.

The mobilization phase commences at bud swell and continues until growth ceases in mid-summer. From bud swell to flowering protein hydrolysis occurs in the storage tissue of older shoots and branches and soluble nitrogen (principally aspartic acid, asparagine and glutamine) is translocated from these tissues to the developing meristems. Once the air temperature rises to 40^{o} to 45^{o} F mobilization of root and rootstock reserves commences followed by its translocation to the shoots. These root and rootstock reserves quickly replace the reserves of the older shoots

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as the main source of nitrogen for the developing meristems. Next, prior to the abscission of the flowers which have failed to develop (only a small portion of the total number of flowers per tree develop into fruit), one-third to one-half of their nitrogen is translocated back into the shoots mainly in the form of free amino acids. Finally nitrogen absorbed by the roots during the spring may be translocated to the shoots to supplement that available from the storage reserves. This is more apparent in trees with poorly developed reserves than in trees that have been well supplied with nitrogen. Under conditions of adequate nitrogen supply, much of the nitrogen absorbed by the roots during the spring appears to be used to replenish depleted reserves rather than be directly involved in growth.

Three aspects of the annual nitrogen rhythm in deciduous fruit trees will now be considered in detail in relation to the use of foliar sprays, namely fruit set in relation to summer, autumn and spring sprays; fruit and shoot growth in relation to spring sprays; and the site of nitrate reduction in fruit trees in relation to nitrate sprays. A fourth aspect, whether soil applications which are essential to the build up of root and rootstock nitrogen reserves, can be partly or wholly replaced by foliar-applied nitrogen, will be considered in the review of tree response to foliar-applied nitrogen.

Fruit Set

The role of nitrogen in fruit set (i.e. in the growth of young fruit immediately after fertilization--growth which occurs by cell division only) is well established in the literature (Taylor 1969). Nitrogen is essential to the cell division process in deciduous fruit and this nitrogen comes from the reserves accumulated in the woody storage tissue the previous season, especially in the autumn. As examples of the influence of nitrogen on cell division Reeve and Neufeld (1959) found with the Elberta peach that high nitrogen fruit of a given size had more but smaller parenchyma cells than low nitrogen fruit and Hosoi and Ishida (1964) found that cell division was markedly reduced in nitrogen-deficient pear fruit. That the source of this nitrogen for fruit set is the shoot and root storage reserves created the previous season was well demonstrated by Hill-Cottingham and Williams (1967). Nitrogenous fertilizer was applied to apple trees only during spring or summer or autumn. The following spring only summer and autumn treated flowers had ovules that remained viable for six days after anthesis; this was the minimum period found necessary for the pollen tubes to effect fertilization. An earlier experiment (Hill-Cottingham 1963) had shown that fruit set on young apple trees was dependent on the level of nitrogen supplied to the rootstocks in the previous year.

One possible role for foliar applications of nitrogen then is to increase the amount of nitrogen available to the fruit during fruit set. As indicated above this nitrogen would normally come from shoot storage tissue and as these reserves are depleted it is drawn from root and rootstock reserves. Spray applications during and just after bloom (two pink, a calyx, and a first cover spray) have been advocated by Fisher and co-workers (Fisher et al. 1948; Fisher and Cook 1950; Fisher 1952) to supplement tree reserves during fruit set. Difficulties with spray applications at this stage were that there was very little leaf area available for absorption and spray concentrations had to be kept low to avoid injury to buds and young leaves.

Oland (1960) recommended one or more nitrogen foliar sprays be applied between fruit harvest and leaf fall, to build up the nitrogen stored in both shoots and buds in the autumn for the following spring. He found sprays could be applied in autumn at seven to eight times the concentration possible in spring. However great variations, from 20 to 80 per cent (Taylor 1967a), may occur in the amount of nitrogen which migrates from the leaves back into the shoot prior to leaf abscission. The foliage of a mature apple tree may contain up to one-half of the nitrogen content of the tree at the end of the growing season (Murneek 1942), and translocation from leaves to woody shoots commences three to four weeks prior to

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abscission (Oland 1963b). On the average about 40 per cent of the nitrogen is reabsorbed (Murneek 1930), but under windy conditions causing premature leaf abscission this may be considerably reduced (Oland 1963b, c). In one study post-harvest sprays increased the incidence of premature leaf abscission (Delver 1966). Another disadvantage of post-harvest applications, rarely referred to in the literature (e.g. Murneek 1930), is that much of the nitrogen applied as a spray and subsequently stored may be lost during pruning.

In a third approach to increasing the nitrogen available for fruit set in apple trees, Williams and Rennison (1963) advocated summer foliar applications made once shoot growth had ceased. An added advantage of such sprays might be the forcing of undifferentiated buds to differentiate into flower rather than leaf buds where this might be desirable (e.g., when wishing to force a young tree into production). They suggested two possible disadvantages of the method, viz. that if applied too early the shoots may be forced into new growth subject to winter injury and more importantly that fruit maturity may be delayed.

Fruit and Shoot Growth

Boynton and Oberly (1966) have explained the effect of nitrogen on fruit growth as follows. Where nitrogen

has caused bloom and set to increase in greater proportion than vegetative growth and leaf efficiency, nitrogen fertilization may cause a decrease in average fruit size at harvest, but where there is no great increase in bloom or set or where the increase in vegetation and leaf efficiency more than compensate for the increase in bloom and set, there may be a marked positive effect of nitrogenous fertilizer on fruit size.

Thus in seasons of heavy fruit set the nitrogen reserves of a tree may be inadequate to provide sufficient vegetative growth to ensure sizing of the crop. If the additional fertilizer necessary is applied as a foliar spray it should be available to the meristems more rapidly and its effects should be of less duration (thus reducing the danger of delaying fruit maturity) than it would if applied to the soil (Fisher 1952). The use of nitrogen foliar sprays in conjunction with the cover sprays in late spring and early summer has been the only approach to foliar spraying with nitrogen widely accepted commercially.

The Site of Nitrate Reduction in Fruit Trees

It is generally accepted that the site of nitrate reduction in deciduous fruit trees is mainly in the roots (Nightingale 1937, Bollard 1953a), which immediately raises the question whether fruit trees would be able to metabolise and assimilate in their leaves foliar sprays

containing nitrate ions. However a detailed examination of the evidence strongly suggests that they would.

Thomas (1927a, b) assayed for nitrate and nitrite in various parts of a mature apple tree through an annual cycle and while finding nitrate ions principally in fine roots also found them in leaf buds as they were opening. Eckerson (1931) determined the nitrate reductase activity of various parts of a mature Stark apple tree at weekly intervals for one year. She found high nitrate reductase activity during autumn and winter in the fine roots and during spring in both the fine roots and the buds and adjacent bark. Nitrate ions were always found in buds at bud swell but not in the leaves when they opened. However there was a very low but detectable level of nitrate reductase in the leaves throughout their cycle except for three weeks in late spring. Activity in the leaves reached a peak at the end of June and again at the end of July. She also found that, although apple trees do not ordinarly contain nitrate, two trees which had received heavy nitrate applications shortly after the flowers had opened contained nitrate ions in leaves at the five foot level after three days. Within a week all the nitrate had disappeared and no more was found in the leaves that season. Stuart (1932) reported similar results. When sodium nitrate was supplied in extremely high amounts to the roots of small apple trees, nitrate ions appeared in the leaves (0.037 per cent on a dry weight basis).

In summary this early evidence indicated that in apple trees reduction of nitrate and synthesis of amino acids takes place mainly in the roots, however nitrate ions are translocated, unreduced, to the buds during bud burst or to the leaves in the exceptional case of excess nitrate application. The nitrate reduction data together with the rapid disappearance of nitrate from the apple leaves was evidence of the ability of apple leaves to metabolise nitrate. This latter point was not conclusive though, for as Beevers and Hageman (1969) have pointed out, the nitrate reduction data may have represented non-enzymatic conversion of nitrate to nitrite in Eckerson's (1931) study, because even boiled plant extracts effected the conversion.

More recent research has however confirmed the early evidence. Bollard (1953a, b, 1957a) failed to find any nitrate ions in tracheal sap extracted from one-year-old shoots of mature apple trees at any time during the annual rhythm. This also held true for other commercial stone and pome fruit trees (Bollard 1957b). Subsequent work on the nitrogen constituents of, and storage and mobilization of nitrogen in apple (Oland 1959) and peach trees (Taylor 1967b; Taylor and May 1967; May and Taylor 1967; Taylor and van den Ende 1969) confirmed the absence of nitrate ions in scion tissues under normal conditions. In confirmation of root reduction Grasmanis and Nicholas (1967) extracted an active nitrate reductase from apple roots but

were unable to determine its physiological electron donor, then Klepper and Hageman (1969) extracted from apple roots a typical NADH[1]-dependent nitrate reductase using insoluble polyvinylpyrrolidone in the extraction medium.

Klepper and Hageman (1969) also added excess nitrate ions to the root medium of apple seedlings and subsequently found nitrate ions and both nitrate and nitrite reductase enzymes in the leaves and nitrate ions and nitrate reductase in the stems and petioles. They found trace amounts of nitrate and nitrate reductase in leaves of mature apple trees (the trees had received low annual amounts of nitrogen), and were able to induce the enzyme in excised leaves by soaking them in 0.1 M potassium nitrate for four hours. This seems conclusive evidence that apple leaves can metabolise nitrate ions.

The situation in other plant species which normally reduce nitrate in their roots appears to be similar to that found in apple trees. Wallace and Pate (1965, 1967) found that in the field pea all nitrate was metabolised in the roots and only reduced nitrogen was found in exuded sap, provided the supply of nitrate to the roots was normal. However, when the plants were supplied with nitrate levels

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[1]Reduced nicotinamide adenine dinucleotide, the physiological electron donor for the enzyme.

higher than 10 p.p.m., nitrate was transported to the shoot and nitrate reductase was induced.

In conclusion it seems probable that in Prunus spp. leaves are able to metabolise nitrate even though roots normally reduce all the nitrate ions the trees absorb. Nevertheless the presence of a nitrate reducing capacity in stone fruit leaves remains to be demonstrated.

The Response of Fruit Trees to Nitrogen Foliar Sprays

The form of nitrogen most studied as a foliar spray for fruit trees has been urea. It is the most concentrated source of nitrogen available (46 per cent nitrogen) for spray application and is absorbed and metabolised rapidly by many crops (Wittwer and Teubner 1959; Wittwer 1967). It has proved to be an effective partial substitute for soil-applied nitrogen in commercial practice for apple trees and several Citrus species but not for pear or stone fruit (Prunus spp.) trees.

Urea Sprays

Apple Trees.--Urea sprays have been used commercially both to supplement soil nitrogen applications and to supply the total nitrogen requirement of apple trees. It is well established both through research and in commercial practice that urea sprays applied in late spring (e.g. at petal fall and with the first two cover sprays)

at low rates (e.g. 0.6 per cent) are a valuable supplement to and allow a reduction in soil nitrogen applications (Hamilton et al. 1943; Fisher et al. 1948; Fisher and Cook 1950; Proebsting 1951; Fisher 1952; Blasberg 1953; Norton and Childers 1954).

Fisher's (1952) and Blasberg's (1953) studies indicated that these spring sprays made nitrogen readily available to shoot meristems and developing fruit which otherwise had to come from rootstock reserves or via root absorption of soil nitrogen, two processes which are particularly tardy when cool weather is experienced in spring. Trees responded more rapidly to the sprays than to comparable soil applications but the effect was of shorter duration. Thus fruit set and development were enhanced without the disadvantage of delayed fruit maturity often characteristic of soil applications in late spring.

Use of urea foliar sprays to supply the total nitrogen requirement of apple trees was suggested (e.g. Benson 1953) and was adopted commercially particularly in Vermont and New York (Forshey 1963). However this practice has not been successful on a long term basis (Beattie 1958; Forshey 1963). Forshey (1963) found that having received all their nitrogen in the form of urea foliage sprays for several years, apple trees became unexplainably low in vigor. Leaves were large and dark green and leaf analysis indicated satisfactory levels of nitrogen. Yet terminal

shoot growth was poor, most lateral buds failed to open, both flowering and fruit set were unsatisfactory, and shoot bark analyses indicated a nitrogen deficiency. He studied nitrogen translocation from leaves sprayed with urea using trees growing in sand culture and found that while translocation from the leaves occurred in late summer, almost all the nitrogen was transported to the young shoots. There was negligible net movement to storage tissues. Thus it would appear that soil nitrogen applications are essential to the maintenance of an apple tree's nitrogen reserves. Foliar urea sprays can only be a supplement to soil applications not a replacement for them.

Post-harvest urea sprays have aroused research interest in Europe. Oland (1960, 1963a, 1966) reported that 4 per cent urea sprays applied just after fruit harvest increased the organic nitrogen content of leaves by 51 per cent after two days and of spurs with developing flower buds by 31 per cent, and of terminal shoots by 16 per cent by mid-November (Norway). Mid-October sprays resulted in yield increases of 50 per cent the following season. These yield increases were due to more fruit and not to larger fruit. However soil applications of calcium nitrate in autumn and spring gave comparable yield increases.

Studies of post-harvest urea sprays in England (Little et al. 1966; Wilson 1966) showed no yield increase

from a 4 per cent spray. The authors ascribed this to: (a) their trees having an adequate nitrogen status initially, whereas Oland's trees were below normal in nitrogen; and (b) to Oland's trees being very much larger than theirs thus having a much greater absorption area. Delap (1967) found that a post-harvest 4 per cent urea spray increased the nitrogen content of leaves and prunings and increased fruit set the following season on 2-year-old trees of both low and high nitrogen status. However he considered it unlikely that the treatment would have appreciably affected cropping in a well managed orchard. Delver (1966) found one undesirable result of post-harvest sprays was premature leaf fall immediately after spray application.

Williams and Rennison (1963) used summer urea sprays as a means of brining vigorous young trees into crop. In their experiments three summer sprays of 1 per cent urea applied once extension growth had ceased successfully brought vigorous young trees into crop and increased fruit yield the following season. However the sprays were less effective than corresponding soil applications of either 117 lb. or 235 lb. nitrogen per acre.

Citrus spp.--Most Citrus spp. respond favorably to urea sprays. These sprays were first tested on lemon seedlings (Haas 1949) and have been extensively studied for orange trees (Jones and Parker 1949; Jones and Steinacker 1953; Jones et al. 1957; Beutel 1962; Labanauskas et al.

1963). Jones and Embleton (1965) have reviewed these and other studies, and recommend using urea at 0.9 per cent. The urea used should contain no more than 0.25 per cent biuret to avoid biuret toxicity (Jones 1954; Jones and Embleton 1954). The sprays may be applied at four-weekly intervals to bearing orange and grapefruit trees in winter and spring, particularly during flowering and fruit setting, and to non-bearing trees any time there is a need for nitrogen.

Jones and Embleton (1965) estimated that if the total annual nitrogen needs of a tree were to be supplied by urea foliar sprays then at least three sprays (at 0.9 per cent) would be necessary as one spray at this concentration on an average size tree supplies ca. 0.25 pounds of nitrogen. However they gave no evidence that urea sprays could solely maintain mature trees in good vigor for several seasons. Commercially the tree's nitrogen requirement is often supplied partly as a foliar spray and partly as a soil dressing, the proportion depending on costs.

Prunus spp.--Peach, sour cherry and sweet cherry trees have been reported to respond to spring foliar applications of urea but only at high concentrations (e.g. 1.8 per cent). However, at these concentrations the trees often suffered from biuret toxicity (Weinberger et al. 1949; Anon. 1951; Proebsting 1951; Bullock et al. 1952; Benson 1953; Ticknor 1953; Eckert and Childers 1954; Norton and

Childers 1954; Walker and Fisher 1955). Eckert and Childers (1954) and Norton and Childers (1954) concluded that the foilage of Elberta peach absorbed limited amounts of nitrogen from spring sprays of 1.8 to 2.4 per cent urea. These sprays increased leaf nitrogen concentration measurably, were apparently metabolised, and caused shoot terminal growth to increase. The use of hydrated lime with urea to reduce biuret injury, of sodium bentonite wetting agent and of molasses to raise the carbon to nitrogen ratio, did not give consistently better results than urea alone. Dormant sprays of urea from 1.2 to 12 per cent had no effect on leaf nitrogen content or tree growth.

Apricot (Proebsting 1951; Benson 1953), almond (Anon. 1951; Proebsting 1951; Norton and Childers 1954) and plum trees (Proebsting 1951; Wlodek et al. 1960; Abdelal and El-Tomi 1965; White and Glen 1967) have shown no response to urea sprays applied to the foliage in spring. Almond trees are subject to urea injury at low concentrations (0.6 per cent) (Proebsting 1951; Norton and Childers 1954). Pear and quince trees have shown a lack of response to urea sprays similar to that of stone fruit trees (Benson 1953; Abdelal and El-Tomi 1965).

Nitrogen Sprays other than Urea

Nitrogen sprays other than urea have received limited testing on apple, citrus, and peach trees.

However no marked beneficial response to the nitrogen has been obtained.

Nitrate Sprays.--Apple tree response to spring sprays of sodium and potassium nitrate has varied. Low concentrations (0.6 per cent) have caused injury (Hamilton et al. 1943) yet higher concentrations (up to 3 per cent) have been applied without injury (Fisher and Walker 1955; Raffer 1968). Summer foliar sprays of calcium nitrate have been widely used (usually three 1 per cent sprays at fortnightly intervals) to correct calcium deficiency in apple fruit, particularly "Bitter Pit" disorder. These sprays have generally succeeded in increasing the amount of calcium in leaves and, in some instances, fruit. However, as they have rarely caused any delay in fruit maturity, it seems doubtful that significant quantities of nitrogen have been absorbed from them (Baxter 1960; Beyers 1962, 1963; Martin et al. 1965, 1967; Stevenson 1967).

Magnesium deficiency in Citrus spp. particularly orange trees has been repeatedly corrected by an annual foliar spray of 1 per cent magnesium nitrate (often prepared by mixing 10 pounds of magnesium sulphate and 10 pounds of calcium nitrate per 100 gallons for economy) applied once the spring growth flush was two thirds expanded at rates of 600 to 1,000 gallons per acre according to tree size (Embleton and Jones 1959; Strauss 1963a, b; Bar-Akiva 1965; Calvert and Reitz 1966; Bar-Akiva and Kaplan 1967).

Bar-Akiva (1965) found that combining zinc and manganese with magnesium nitrate improved zinc and manganese absorption.

Potassium deficiency in orange, lemon, and mandarin trees has similarly been corrected using a 3 to 5 per cent potassium nitrate spray following the spring growth flush at monthly intervals (Page et al. 1963; Embleton et al. 1964; Cutuli 1966; Bar-Akiva and Kaplan 1967). Cutuli's (1966) study indicated that potassium absorption was not being accompanied by significant nitrate absorption, however this conclusion was based on leaf concentration data, not absolute amounts of nutrients present, and may be misleading.

On peach trees, spring foliar sprays of sodium and potassium nitrate at 0.6 per cent have proved neither beneficial nor injurious (Hamilton et al. 1943; Norton and Childers 1954). However Norton and Childers (1954) reported that a zinc nitrate spray at 0.24 per cent seriously injured peach foliage even in the presence of lime.

Foliar applications of potassium nitrate (Kenworthy 1965) have been reported to be beneficial when peach trees showed symptoms of potassium deficiency and a 5 per cent delayed dormant (i.e. green tip) spray has been reported to be helpful in recovery of trees from winter injury. These reports were based on unpublished field observations and the general application of such sprays was not recommended.

Ammonium sprays.--Ammonium sulphate sprays at low concentrations (up to 0.6 per cent) have not been beneficial to either apple or peach trees (Hamilton et al. 1943; Norton and Childers 1954).

Possible Reasons for the Anomalous Response to Nitrogen Foliar Sprays in Prunus spp.

It is now clear that nitrogen foliar sprays (mainly 0.6 per cent urea sprays) applied in spring and early summer to bearing apple trees, may be used to supplement soil nitrogen applications particularly by providing any additional nitrogen which may be needed for fruit set and development. It is also clear that pear, quince, and stone fruit trees will not give a similar, commercially meaningful, response to 0.6 per cent urea sprays. Thus it seems that while these species may absorb some foliar-applied urea, they are not as efficient in this respect as apple trees.

For a foliar-applied chemical to be utilized by a tree it must first be absorbed, then metabolised (assimilated) by the tree. These two processes would normally occur in the leaf itself when urea is the chemical in question (Hinsvark et al. 1953). During the process of leaf absorption of any molecule (Leopold 1964) the molecule must first traverse a lipophilic cuticle, then penetrate the hydrophilic cell wall and enter the apoplast (the aqueous network permeating cell walls). The molecule must then be

transported from the apoplast across the lipoprotein plasmalemma and be deposited in the cytoplasm where it may be metabolised. Depending on the molecule, metabolism will either occur in the cytoplasm itself or in one or more sub-cellular organelles.

Early studies of urea absorption and metabolism in apple leaves (Cook and Boynton 1952; Rodney 1952; Boynton et al. 1953) indicated that apple leaves absorbed urea more rapidly through the lower surface than the upper surface although by the end of a week total absorption by both surfaces was approximately equal. Young expanding leaves were more efficient absorbers than mature leaves, addition of a surfactant to the spray enhanced absorption, and weather conditions favoring low vapor pressure deficit (e.g. high humidity) in general favored absorption. There appeared to be rapid metabolism of urea-nitrogen in treated leaves followed by rapid translocation from them.

Dilley and Walker (Dilley 1960; Dilley and Walker 1961a, b) compared urea absorption and metabolism in peach and apple leaves. They showed that the failure of peach leaves to effectively utilize foliar-applied urea was not due to their inability to hydrolyse or subsequently assimilate urea. When ^{14}C, ^{15}N labelled urea was supplied to excised leaves through the petiole both isotopes were readily incorporated into amino acids, amides and protein within 20 hours. The distribution of ^{14}C and ^{15}N in the

amino acids indicated initial hydrolysis to $^{14}CO_2$ and $^{15}NH_3$ followed by incorporation into amino acids. This conclusion was supported by the isolation of an active urease from both apple and peach leaves. Incidently, urea hydrolysis proceeded to a greater extent in peach than in apple leaves, not the reverse.

In another series of experiments they found that when leaf discs taken from peach leaves previously soaked in acetone for 3 minutes, were incubated with 0.25 M urea for 30 minutes, the rate of urea hydrolysis was double that of discs from leaves that had not received the acetone pre-treatment. They suggested that since the acetone is capable of dissolving certain lipid constituents of the cuticle, the increased enzyme activity was a reflection of more rapid cuticular penetration by the urea. Thus, lipids may be reducing cuticular penetration by urea in peach.

In a third series of experiments they showed that benzaldehyde would inhibit purified urease. They noted that benzaldehyde is found in Prunus species and postulated that, in those species which do not effectively utilize foliar-applied urea, urea passing through the cuticle might pick up aldehydes which subsequently form addition products with urea which urease is unable to hydrolyse. However, the quantities of aldehyde necessary for this to be effective would be considerable.

Further evidence that epicuticular waxes may reduce cuticular penetration of foliar-applied molecules in peach leaves was obtained by Bukovac (1965). Brushing the upper and lower surfaces of peach leaves lightly with a camel's hair brush enhanced uptake of 3-chlorophenoxy-α-propionic acid by 22 and 34 per cent respectively.

Bester et al. (1965, 1967) reported foliar absorption of a 0.5 per cent urea solution by apple, pear, peach and vine plants. In one experiment, a 2 or 5 per cent ^{14}C labelled urea solution (25 microlitres) was applied within a lanolin ring to the first mature leaf of greenhouse-grown peach seedlings. After a three day absorption period the treated area of each leaf was removed and discarded, then the remainder of the plant analysed. They found that the peach seedlings had absorbed and metabolised the urea, total radioactivity and radioactivity in both treated leaves and stems being greater in plants treated with 5 per cent than 2 per cent urea solution. The main labelled amino acids produced were aspartic acid, α-alanine, glutamic acid, γ-amino butyric acid and ethanolamine. Addition of the surfactants (0.004 per cent) Triton B 1946 and Carbowax 4000 did not aid absorption and Teepol 410 and Tween 20 significantly reduced the amount of urea absorbed.

To summarize the evidence on the anomalous response of Prunus spp. to urea sprays, it is clear that both apple

and peach trees can absorb, metabolise and translocate foliar-applied urea. The difference between apple and peach trees then must be a quantitative rather than a qualitative difference. Present indications are that the cuticle of the peach leaf, due to its lipoidal character, is reducing the quantity and/or the rate of urea absorption. This would seem a suitable starting point for investigations of this phenomenon.

STUDIES ON NITRATE METABOLISM IN LEAVES OF *PRUNUS* SPECIES

The question was raised in the Review of Literature whether stone fruit tree leaves could metabolise nitrate ions and thus be able to benefit from nitrate foliar sprays. The evidence indicated that *Prunus* leaves probably could metabolise nitrate ions but this remained to be demonstrated. In the experiments now reported, *Prunus* leaves were supplied with nitrate ions via the petiole then examined for the presence of nitrate reductase, which is a substrate inducible enzyme and the first enzyme in the metabolic sequence from nitrate to amino acids and normally the rate limiting step in nitrate assimilation (Bandurski 1965).

Materials and Methods

Nitrate reductase enzyme was induced and studied in the leaves of young stone fruit trees growing in sand culture and in excised leaves of mature trees.

Plant Material

Sand Culture of Young Trees and Oats.--Apple (*Malus sylvestris* Mill. cv. "Jonathan"), apricot (*Prunus*

armeniaca L. cv. "Goldcot"), sour cherry (Prunus cerasus L. cv. "Montmorency"), sweet cherry (Prunus avium L. cv. "Hiedelfingen"), peach (Prunus persica [L.] Batsch. cv. "Glohaven") and plum (Prunus domestica L. cv. "Stanley") trees, all 2-years old except the apricot trees which were 1-year old, were grown in calcined clay ("Turface", Wyandotte Chemical Corp., Wyandotte, Michigan). They were maintained in moderate vigor and low nitrogen status with a modified Long Ashton solution (Hewitt 1966) of which each tree received 2 litres twice per week. Any additional water requirement was supplied as deionised water. The nutrient solution contained in mM concentrations, $(NH_4)_2SO_4$ 2.5; Na_2HPO_4 1; $MgSO_4$ 1.5; K_2SO_4 2.5; $CaCl_2$ 5.0; and in μM concentrations, Fe^{+3} 50 (as Sequestrene 330 Fe, Geigy Agricultural Chemical Co., Yonkers, New York); $CuSO_4$ 2; $MnSO_4$ 10; $ZnSO_4$ 2; H_3BO_3 30; $(NH_4)_6\ Mo_7O_{24}$ 0.07.

Prior to the commencement of each experiment the trees were brought in a dormant condition from cold storage (38° F) where they had been kept in darkness with occasional watering to prevent drying out, for at least 10 weeks. The trees were pruned to three shoots and grown in a greenhouse with a 16-hour photoperiod of supplemental light (200 ft. candles cool-white fluorescent at leaf level). Temperature in the greenhouse varied between 60° and 90° F and humidity between 45 (day) and 75 (night) per cent. Insect control was achieved by spraying the trees twice a week with Kelthane or Guthion.

Oats (Avena sativa L. cv. "Gary") were grown in the same greenhouse as the trees to provide a readily available and highly active source of nitrate reductase. The oats were seeded and grown in vermiculite, watered with the 15mM nitrate solution as used in the root uptake studies described below (p. 29), and was harvested for enzyme extraction 5 to 8 days after seedling emergence.

Mature Tree Culture.--Leaves were obtained for enzyme induction studies from the following trees growing at the Horticulture Research Center, Michigan State University, during spring 1970: apricot cv. "Curtis", sour cherry cv. "Montmorency", sweet cherry cv. "Hiedelfingen", and plum cv. "Stanley". The trees were 5-years old, were in good vigor and had received 0.5 lb. nitrogen fertilizer per annum.

Methods

Root Uptake Studies.--Nitrate reductase was induced in leaves of young trees and oats following root uptake of nitrate by substituting 5mM KNO_3 and 5mM $Ca(NO_3)_2$ for 2.5mM K_2SO_4 and 5mM $CaCl_2$ respectively in the nutrient solution. After 3 days the most recent fully expanded leaves were collected for enzyme assay. Standard nutrient solution (OmM NO_3^-) control trees were maintained for comparison.

Petiole Uptake Studies.--Nitrate reductase was induced in excised leaves of sand-cultured trees which had not received the nitrate nutrient solution, and in excised leaves of mature trees, following petiole uptake of nitrate. Current season's shoots were cut from the tree at their junction with one year wood using sharp pruning shears. The base of the shoot was immediately submerged in water and 2 inches were cut off the base under water (to prevent air blockage of the xylem). The shoot was transferred to the laboratory with its base still under water. At the laboratory selected leaves with their petiole attached were individually excised under water with a sharp razor blade, then transferred with a water droplet covering the cut end of the petiole to a 10 ml. beaker containing 10 ml. of the appropriate treating solution. The standard induction solution was 10 mM KNO_3 and the control solution was 10mM KCl.

The leaf was then held for the appropriate induction period (the standard period was 6 hours) at 800 ft. candles (fluorescent) with continual air movement provided by a small fan to increase transpiration and thus increase the rate of uptake of the treating solution. The temperature of the treating solution was not controlled and varied between experiments from 23° to 27° C. Under these conditions the greenhouse grown apricot leaves absorbed ca. 5 ml. of treating solution in 6 hours.

Enzyme Extraction.--Nitrate reductase was extracted from all tissues by use of the procedure of Klepper and Hageman (1969). Unless otherwise stated the following procedure was followed. Petioles and midribs were removed from leaves then 0.5 g. fresh weight was cut into small strips (ca. 5mm x 2mm). The tissue was ground in a mortar with 10 g. hydrated insoluble polyvinylpyrrolidone (Polyclar A.T. - General Analine and Film Corporation, Dyestuffs and Chemical Division, 140 West 51 Street, New York, New York), 10 ml. extraction medium, and ca. 2 g. sand. The extraction medium was a mixture of 50mM potassium phosphate, 5mM ethylene diamine tetraacetate (Na salt), and 10mM cysteine, adjusted to pH 8.8 with KOH. The slurry was pressed through 8 layers of cheesecloth then the filtrate was centrifuged at 27,500 x g. for 15 minutes. The supernatant (enzyme crude extract) was used for nitrate reductase, nitrate and protein assays. The temperature was maintained at 0^{o} to 4^{o} C throughout the extraction procedure. In the case of sour cherry and sweet cherry tissue 15 ml. extraction medium per 0.5 g. tissue was normally required as the slurry became very sticky during grinding.

Assays.--Nitrate reductase was normally assayed using reduced nicotinamide adenine dinucleotide (NADH) as the electron donor. The procedure of Hageman and Flesher (1960) was followed. The reaction mixture comprised: 0.5 ml. of 0.1 M potassium phosphate buffer, pH 7.5; 0.1 ml. of 0.1 M KNO_3; 0.1 ml. of 1.0mM NADH; 0.1 to 0.3 ml.

enzyme crude extract; and distilled water to make a final volume of 1.0 ml. The reaction mixture was incubated at 27° C for 15 minutes and the reaction was stopped by adding the diazo coupling reagents 1 ml. of 1 per cent (w/v) sulphanilamide in 3N HCl plus 1 ml. of aqueous 0.02 per cent (w/v) N(1-naphthyl) ethylenediamine di hydrochloride. The color was allowed to develop for 5 minutes before centrifuging at 1,500 x g. for 10 minutes and the optical density was read at 540 mμ . using a Beckman Model B spectrophotometer. The reference blank was a duplicate reaction mixture stopped at zero time. The nitrite content was obtained from a standard curve and enzyme activity was expressed as mμ . moles NO_2^- formed per g. fresh weight (or mg. protein) per hour.

Nitrate reductase was also assayed using reduced flavin mononucleotide ($FMNH_2$) as the electron donor basically by the method of Paneque et al. (1965) as modified by Klepper and Hageman (1969). The procedure differed from that described above for NADH by replacing the NADH and part of the distilled water in the reaction mixture with 0.1 ml. of 2.4mM flavin mononucleotide (FMN) and 0.05 ml. of 0.08 to 0.10 per cent sodium dithionite ($Na_2S_2O_4$) in 0.01 M potassium phosphate buffer, pH 7.5, respectively. The tubes were sealed with rubber serum stoppers and partially evacuated with a hypodermic syringe. This was found to reduce oxidation at the air-liquid interface during

incubation. The reaction was commenced by shaking each tube gently till the FMN (yellow) was reduced to $FMNH_2$ (colorless). The reaction was stopped prior to adding the diazo coupling reagents by shaking each tube vigorously until the dithionite was completely oxidised as indicated by the reappearance of the yellow color. This was necessary to prevent reduction of the diazo complex by excess dithionite.

Protein was determined on a 2 ml. aliquot of the enzyme extract. The protein was precipitated with 5 ml. of 10 per cent trichloroacetic acid, then the precipitate washed twice with 5 ml. of 10 per cent trichloroacetic acid. The precipitate was dissolved in 1 ml. of 1N NaOH then protein was estimated by the biuret method (Gornall et al. 1949).

Nitrate was assayed by the procedure of Lowe and Hamilton (1967). The soybean nodule bacteroids were prepared from field grown soybeans. The reaction mixture comprised: 0.5 ml. of 0.1M potassium succinate buffer, pH 6.8; sufficient distilled water to make a final volume of 1 ml.; 0.05 to 0.3 ml. of enzyme crude extract; and 0.1 ml. of bacteroid suspension. The reaction mixture was incubated at 45^{o} C for 20 minutes. The reaction was stopped and color was developed and measured as in the NADH-nitrate reductase assay.

Statistical Analyses.--Where appropriate data were analysed by standard statistical procedures (Steel and Torrie 1960) and treatment means were compared using Tukey's ω-procedure.

Results

Nitrate Reductase in Leaves of Young Trees.

Three days after excess nitrate ions were supplied to the roots of young apricot, sour cherry, sweet cherry and plum trees (Table 1), both nitrate and nitrate reductase were found in the leaves. Apricot had three to four times the enzyme activity of the other species. Peach trees, on the other hand, attained leaf nitrate concentrations similar to those of the other species but no leaf nitrate reductase activity could be detected. Apple trees were found to have leaf nitrate and enzyme levels similar to those of the stone fruit trees.

Leaf nitrate reductase activity was found to be negatively correlated with leaf nitrate concentration in apricot (Figure 1.). This was not expected as it was thought leaf nitrate had induced enzyme synthesis. The induction hypothesis was further tested in an experiment in which some trees were treated with a OmM NO_3^- solution and others with a 15mM NO_3^- solution (Table 2). It was found that the 15mM NO_3^- treatment increased leaf nitrate reductase activity without increasing leaf nitrate

Table 1.--Nitrate reductase activity and nitrate concentration of leaves of young apple and stone fruit trees.[x]

Species	Nitrate reductase activity	Nitrate concentration
	(mμ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1})	(mμ moles, g. fresh weight^{-1})
Apple	596 ± 135[y]	708 ± 343
Apricot	1823 ± 541	522 ± 224
Sour cherry	460 ± 245	435 ± 448
Sweet cherry	474 ± 94	402 ± 216
Peach	0	437 ± 291
Plum	408 ± 93	513 ± 303

[x]Trees were grown in sand culture and received a 15mM nitrate nutrient solution. Enzyme was extracted in the presence of 20 g. hydrated PVP. per g. fresh weight of leaf tissue. Extraction medium comprised: 50mM K_2HPO_4; 5mM EDTA; 10mM cysteine; pH 8.8. Nitrate reductase was assayed using NADH as electron donor.

[y]Mean and confidence limits: $\bar{x} \pm t_{0.05} s_{\bar{x}}$

Figure 1. Relationship between nitrate reductase activity and nitrate concentration in leaves from young apricot trees.

**r, the correlation coefficient, is significant ($P < 0.01$).

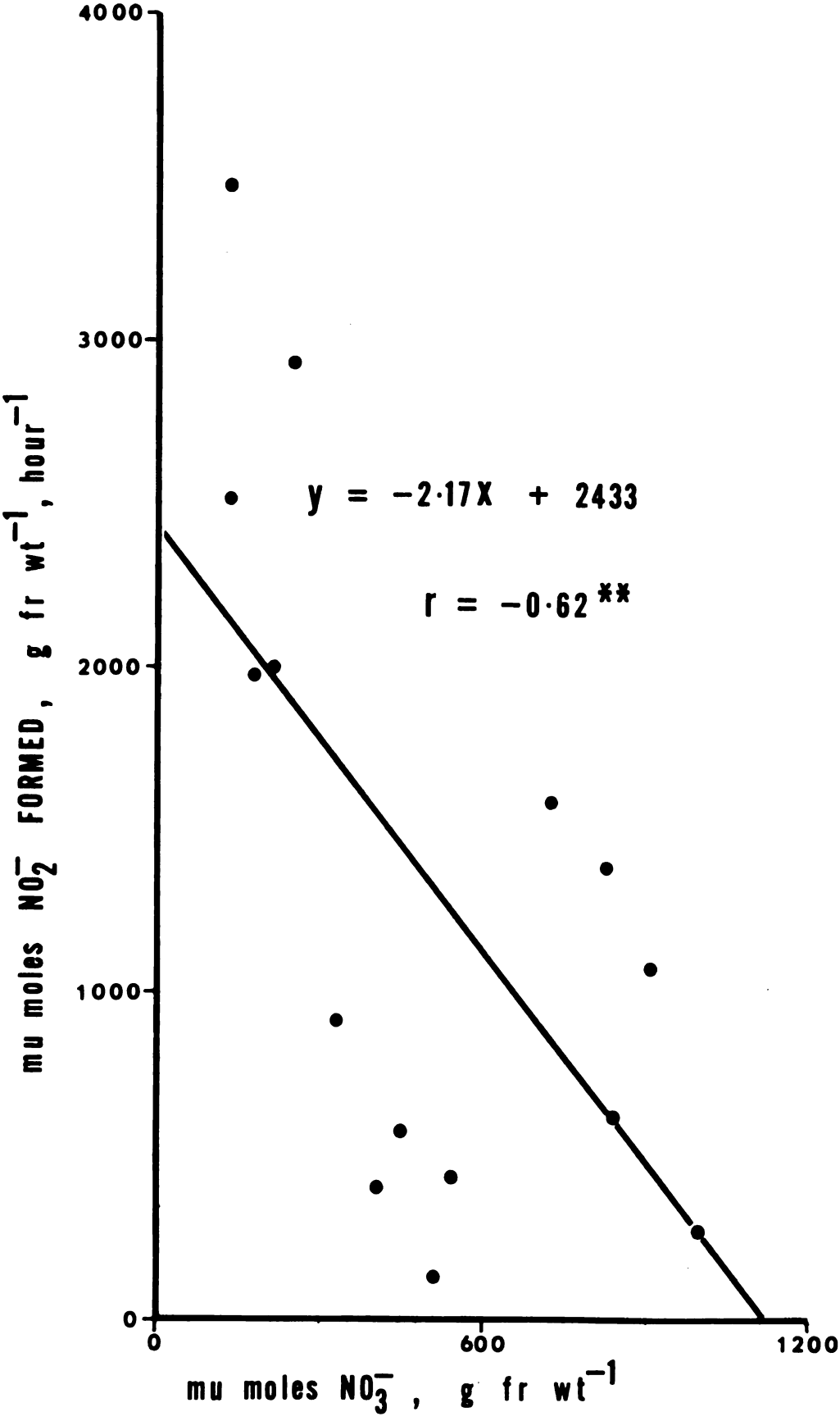

4000
3000
2000
1000
0
0
600
1200
y = −2·17X + 2433
r = −0·62**
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
mu moles NO_3^-, g fr wt^{-1}

Table 2.--Effect of 15mM nitrate nutrient solution on nitrate reductase activity and nitrate concentration of leaves of young apricot trees[x].

Nutrient solution	Nitrate reductase activity	Nitrate concentration
	(mμ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1})	(mμ moles, g. fresh weight^{-1})
OmM NO_3^-	526 a	525 a
15mM NO_3^-.	2228 b	775 a

[x]Means in each column followed by unlike letters differ significantly ($P < 0.01$).

concentration. However when the same experiment was repeated on apple trees (Table 3) leaf nitrate concentration was increased without increasing leaf nitrate reductase activity. The relatively high levels of enzyme activity found in both species in the absence of applied nitrate (ca. 500 mμ.moles/g./hour) is noteworthy.

The inducible nature of nitrate reductase was confirmed using excised apricot leaves. Apple leaves were again included in the experiments for comparison. In time-course studies apricot leaves attained maximum enzyme activity after 6-hours induction (Figure 2) which was also the optimum induction period for apple leaves (not shown). In substrate concentration studies apricot leaves attained maximum enzyme activity at a nitrate concentration of 10mM (Figure 3). Apple leaves again gave similar results (Figure 4). Two sources of apple leaves were available, one having a higher initial enzyme level than the other. For leaves with a low initial enzyme level the optimum nitrate concentration was 10mM and for leaves with a moderate initial level it was 5mM. The enzyme activity ultimately attained in the apricot leaves was much higher than that attained in the apple leaves.

$FMNH_2$ as Electron Donor of Nitrate Reductase.--The enzyme extracted from apricot leaves was found to be able to use either reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin mononucleotide ($FMNH_2$) as its

Table 3.--Effect of 15mM nitrate nutrient solution on nitrate reductase activity and nitrate concentration of leaves of young apple trees[x].

Nutrient solution	Nitrate reductase activity	Nitrate concentration
	($m\mu$ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1})	($m\mu$ moles, g. fresh weight^{-1})
0mM NO_3^-	467 a	556 a
15mM NO_3^-.	672 a	1476 b

[x]Means in each column followed by unlike letters differ significantly ($P < 0.01$).

Figure 2.--Time-course for the induction of nitrate reductase in leaves from young apricot trees.

Excised leaves were incubated with their petioles immersed in 10mM KNO_3 for different periods up to 8 hours. A 10mM KCl control, not shown, maintained the zero time activity throughout the experiment. Each point is the mean of 3 replications. F value for treatments was significant ($P < 0.01$).

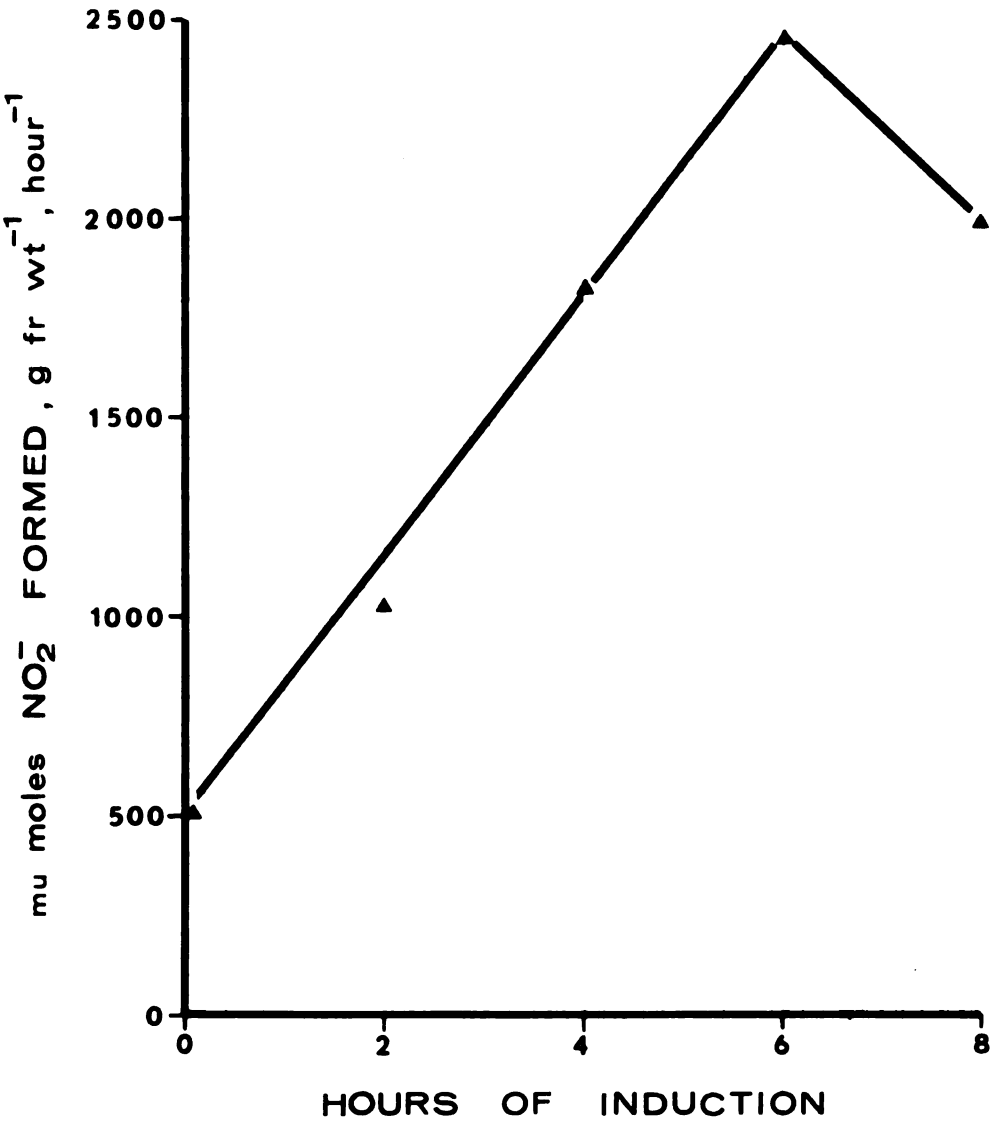

2500
2000
1500
1000
500
0
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
0
2
4
6
8
HOURS OF INDUCTION

Figure 3.--Effect of substrate concentration on induction of nitrate reductase in leaves from young apricot trees.

Excised leaves were incubated with their petioles immersed in KNO_3 solutions of different concentrations for 6 hours. The zero nitrate control was 5mM KCl. Each point is the mean of 3 replications. F value for treatments was significant ($P < 0.01$).

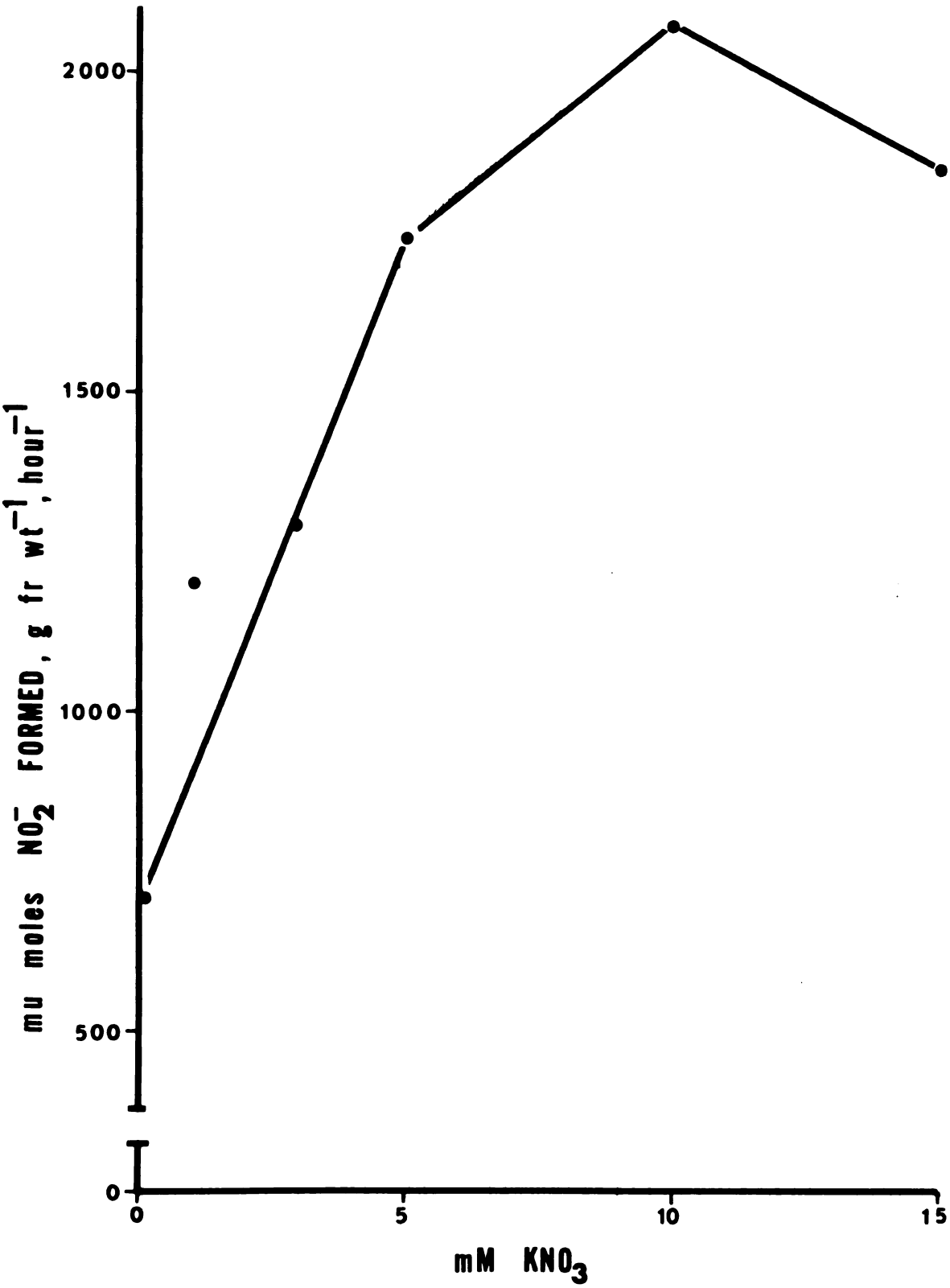
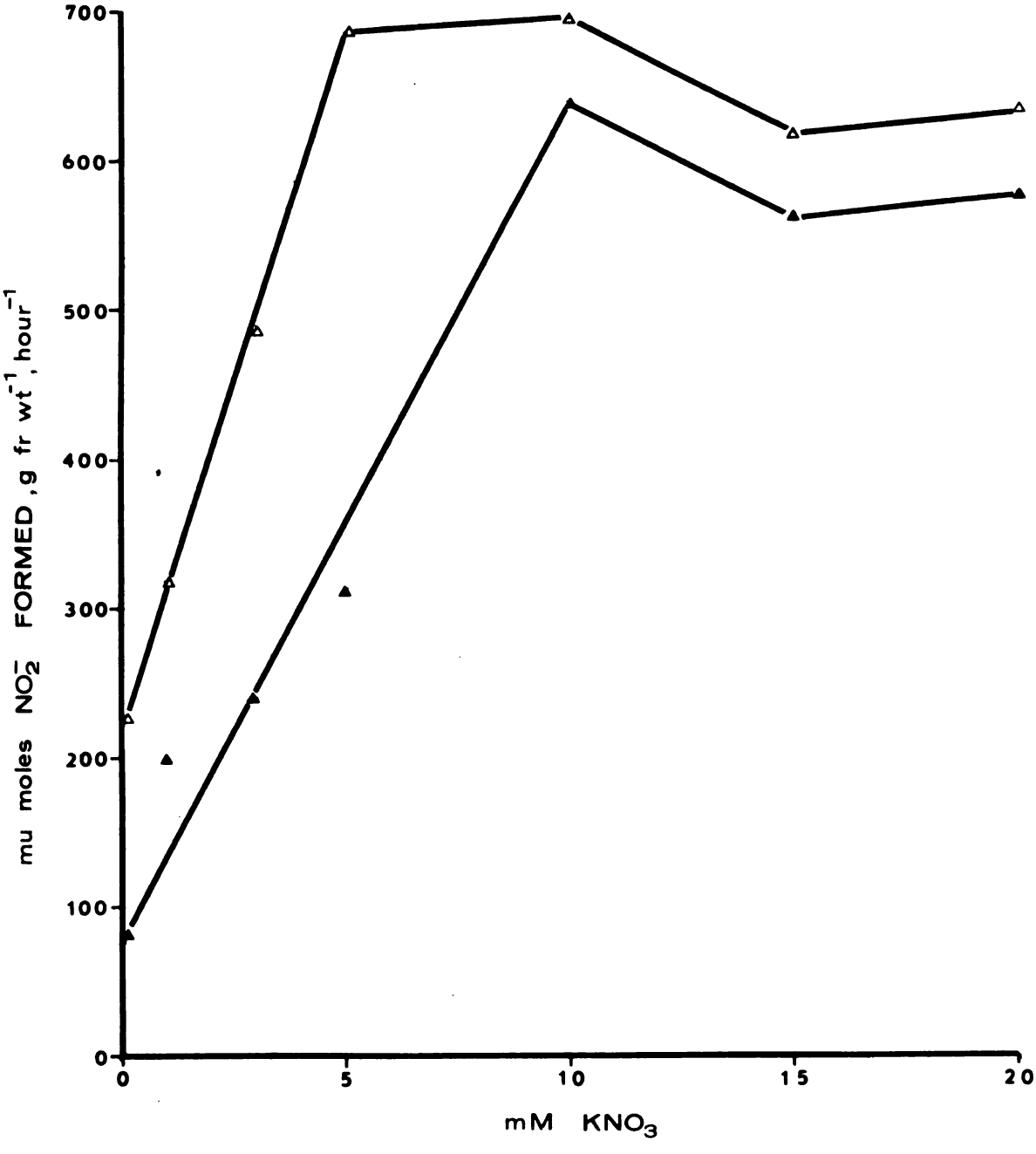

2000
1500
1000
500
0
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
0
5
10
15
mM KNO_3

Figure 4.--Effect of substrate concentration on induction of nitrate reductase in leaves from young apple trees differing in initial level of enzyme activity.
△——△ moderate initial enzyme level
▲——▲ low initial enzyme level.
Excised leaves were incubated with their petioles immersed in KNO_3 solutions of different concentrations for 6 hours. The zero nitrate control was 5mM KCl. Each point is the mean of 3 replications. F value for treatments was significant ($P < 0.01$) in both cases.


700
600
500
400
300
200
100
0
0
5
10
15
20
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
mM KNO_3

electron donor (Table 4). However the activity with $FMNH_2$ never exceeded 40 per cent of the activity with NADH. Similar results were found with an enzyme extracted from an oats control. The admixing of apricot tissue with oats tissue during enzyme extraction did not appear to affect the ability of the oats enzyme to use $FMNH_2$.

Table 4.--Nitrate reductase activity of apricot and oats leaf tissue when assayed using either $FMNH_2$ or NADH as electron donor.

Electron donor	Nitrate reductase activity[x]		
	Apricot	Oats	Oats + Apricot[y]
	($m\mu$ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1})		
$FMNH_2$	745 a	3182 a	3886 a
NADH	2565 b	7214 b	9895 b
		(%)	
$FMNH_2$:NADH	29.0	44.1	39.3

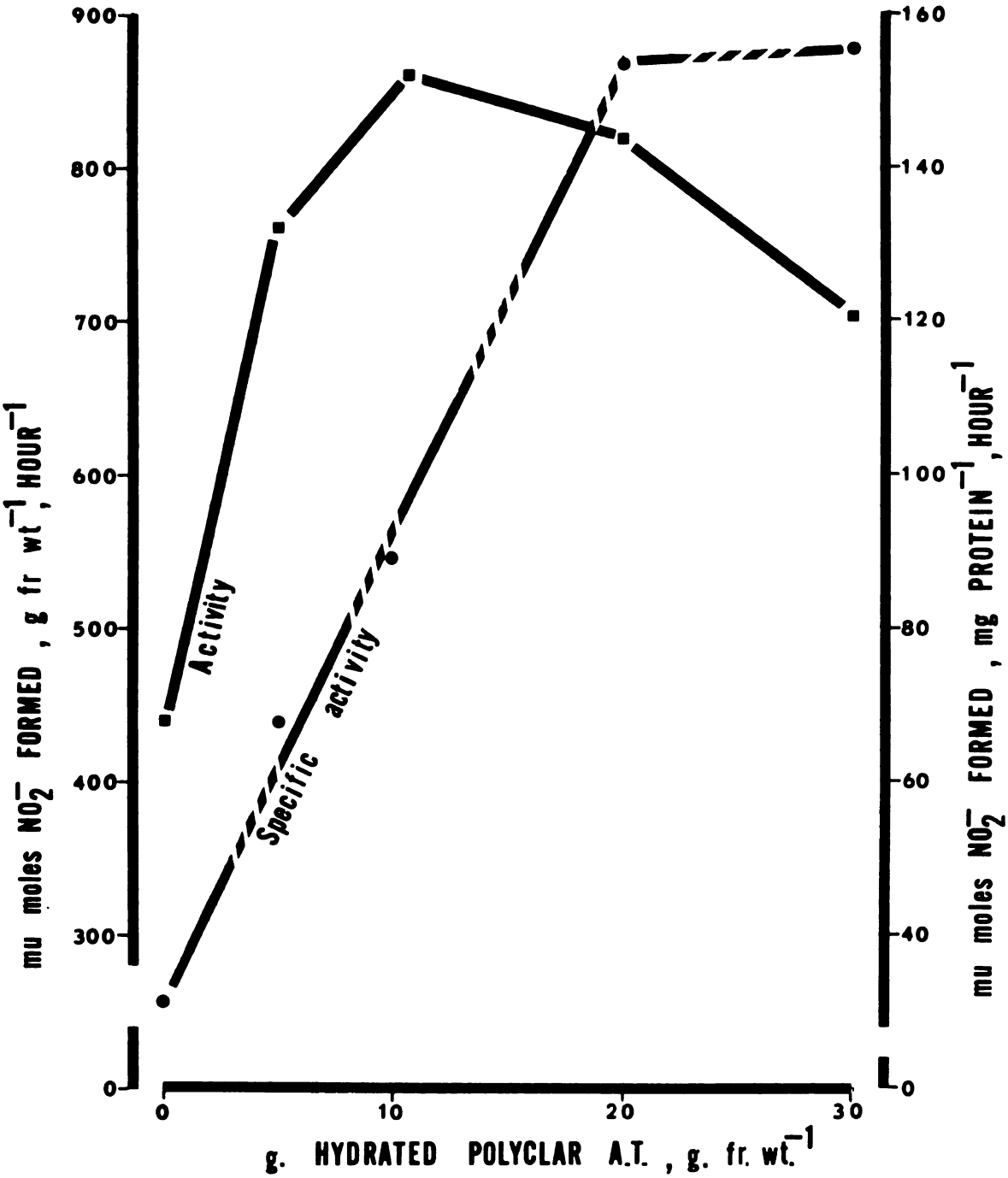
[x]Means in each column followed by unlike letters differ significantly ($P < 0.01$).

[y]0.5 g. Oats tissue extracted in the presence of 0.5 g. apricot tissue.

Extraction with Polyvinylpyrrolidone.--Inclusion of hydrated insoluble polyvinylpyrrolidone (PVP), "Polyclar A.T.", in the extraction medium was found to be essential for optimum recovery of the apricot leaf enzyme (Figure 5). For young leaves a ratio of 10 g. PVP. per g. fresh weight

Figure 5.--Effect of increasing amounts of hydrated insoluble polyvinylpyrrolidone (Polyclar A.T.) on the extraction of nitrate reductase from apricot leaf tissue.

Each point is the mean of 4 replications. F value for treatments was significant for both activity and specific activity ($P < 0.05$).


900
800
700
600
500
400
300
0
160
140
120
100
80
60
40
0
Activity
Specific activity
mu moles NO_2^- FORMED , g fr wt^{-1}, $HOUR^{-1}$
mu moles NO_2^- FORMED , mg $PROTEIN^{-1}$, $HOUR^{-1}$
0
10
20
30
g. HYDRATED POLYCLAR A.T. , g. fr. $wt.^{-1}$

of leaf tissue was sufficient, but older tissue required higher ratios. However, whenever more than 10 g. PVP. was used grinding efficiency was reduced and there was a corresponding tendency for less enzyme to be recovered. It was more convenient to use mechanical homogenisers than a mortar and pestle when more than 10 g. PVP. was needed. But mechanical homogenisers ground leaf tissue less effectively than a mortar and pestle even without PVP. present. Thus there was no net gain in efficiency in their use with large quantities of PVP. It was found that a 20:1 ratio of PVP.:tissue could be conveniently achieved without apparent loss of grinding efficiency by extracting 0.5 g. tissue with 10 g. PVP. in a mortar. Normally 0.5 g. tissue contained adequate enzyme for assay purposes.

The increase in enzyme activity per g. fresh weight and per mg. protein (Figure 5) produced by increasing quantities of PVP. in the extraction medium, was associated with a steady drop in the soluble protein content of the extract (Figure 7). As this differed from published data on apple (Klepper and Hageman 1969) in which protein increased, the experiment was repeated using oats tissue (Figures 6 and 7) with results similar to apricot. Thus with apricot and oats tissue PVP. protected the enzyme from inactivation during extraction, and partially purified it, this purification being more marked than that reported for apple tissue.

Figure 6.--Effect of increasing amounts of hydrated insoluble polyvinylpyrrolidone (Polyclar A.T.) on the extraction of nitrate reductase from oats leaf tissue.

Each point is the mean of 4 replications. F value for treatments was significant for both activity and specific activity ($P < 0.05$).

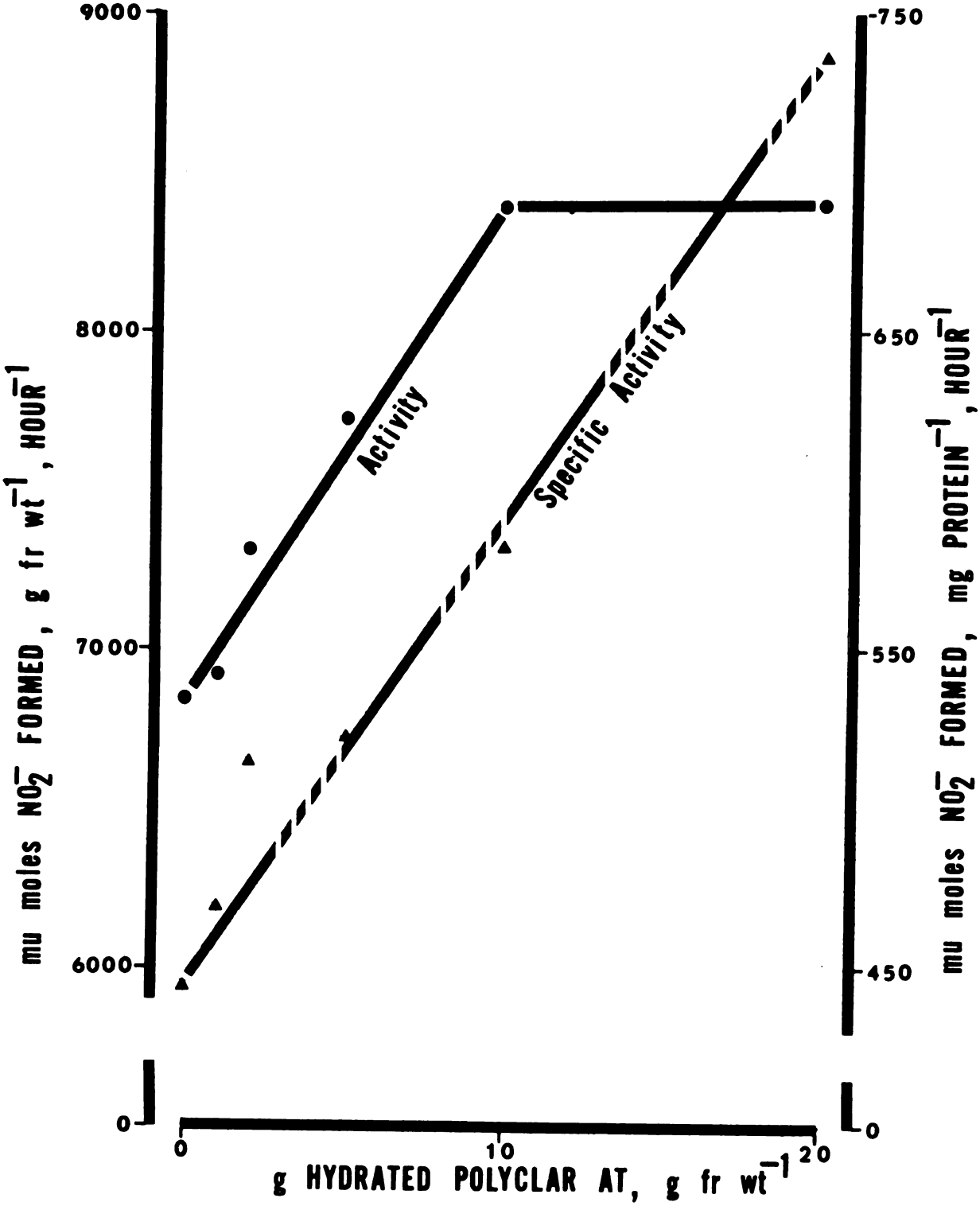
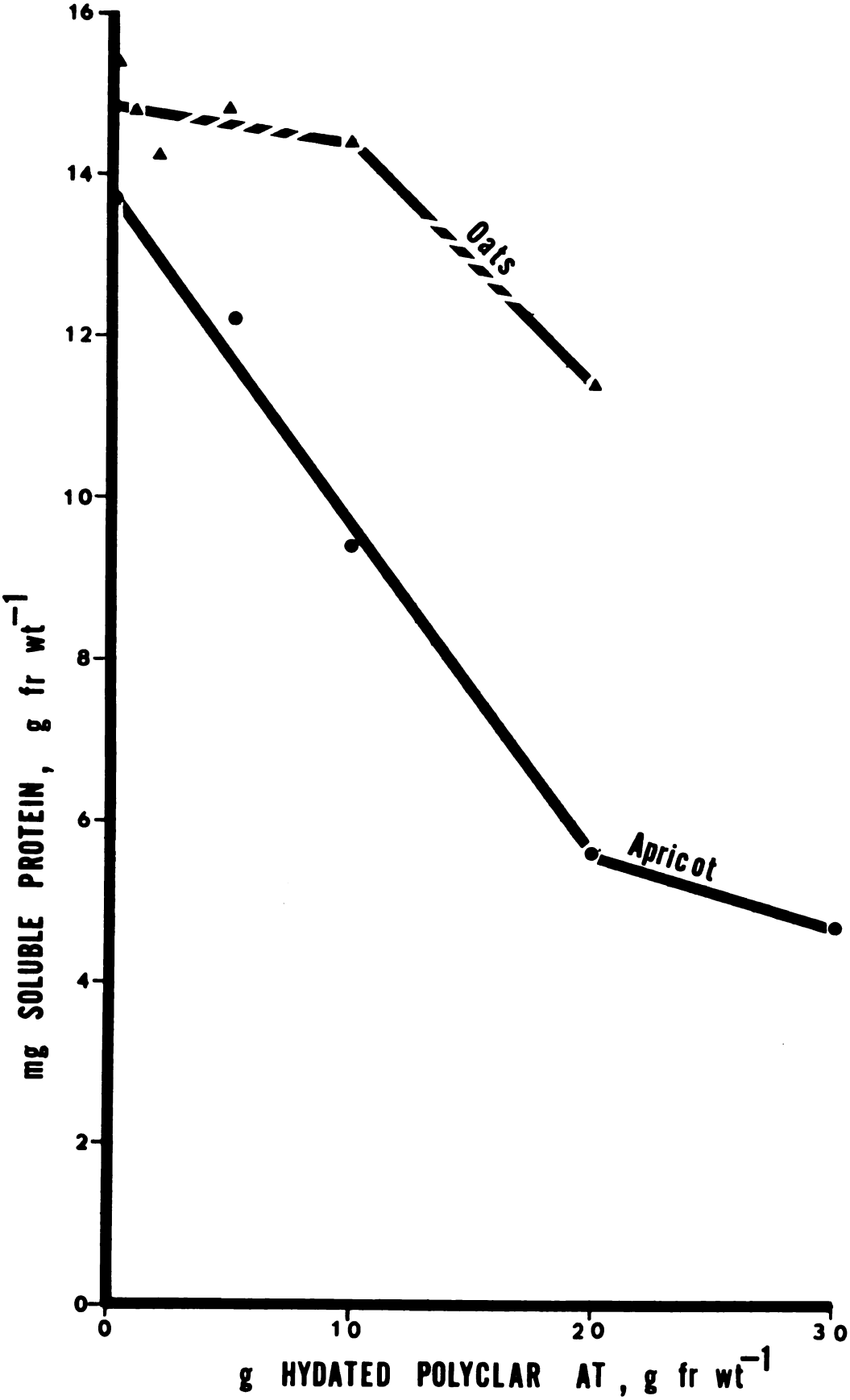

9000
8000
7000
6000
0
750
650
550
450
0
Activity
Specific Activity
mu moles NO_2^- FORMED, g fr wt^{-1}, $HOUR^{-1}$
mu moles NO_2^- FORMED, mg $PROTEIN^{-1}$, $HOUR^{-1}$
0
10
20
g HYDRATED POLYCLAR AT, g fr wt^{-1}

Figure 7.--Effect of increasing amounts of hydrated insoluble polyvinylpyrrolidone (Polyclar A.T.) on the extraction of soluble protein from apricot and oats leaf tissue.

Each point is the mean of 4 replications. F value for treatments was significant ($P < 0.05$) for both apricot and oats.


16
14
12
10
8
6
4
2
0
0
10
20
30
mg SOLUBLE PROTEIN, g fr wt^{-1}
g HYDATED POLYCLAR AT, g fr wt^{-1}
Oats
Apricot

Efficacy of Enzyme Extraction Procedure.--In order to determine whether or not the extraction medium was providing adequate protection for the enzyme during extraction, 0.5 g. oats tissue was extracted either alone or in the presence of 0.5 g. fruit tree leaf tissue (Table 5). Apple, apricot, and plum tissue did not reduce the amount of enzyme recoverable from the oats tissue but sour cherry and sweet cherry reduced it by 50 and 38 per cent respectively, and peach reduced it by 96 per cent. However the reduction caused by sour cherry and sweet cherry tissue was not statistically significant.

Protection of Nitrate Reductase During Extraction from Sweet Cherry and Peach Leaves.--Once the studies of enzyme protection during extraction showed that the extraction medium was not providing protection for the enzyme with peach tissue and was possibly giving only partial protection with sour cherry and sweet cherry tissue, additional PVP. and more powerful reducing agents were tried in the extraction medium.

First oats tissue was extracted in the presence of either sweet cherry or peach tissue with increasing quantities of PVP. It was found that more PVP. was needed for sweet cherry (Figure 8) than had been needed for apricot (Figure 5). Once allowance was made for the amount of PVP. needed to protect the enzyme from inhibitors in the oats tissue (10 g. per g. tissue at the most--Figure 6),

Table 5.--Nitrate reductase activity of oats leaf extracts prepared in the presence of apple and stone fruit leaf tissue compared on a percentage basis with the activity of an extract prepared in the absence of added tissue.[x]

Tissue	Nitrate reductase activity	
	Combined tissue[y]	Added tissue[z]
	(%)	(%)
Oats	100 ab	---
Oats + apple	115 a	6
Oats + apricot	133 a	17
Oats + sour cherry . .	50 b	1
Oats + sweet cherry. .	62 b	0
Oats + peach	4 c	0
Oats + plum	122 a	7

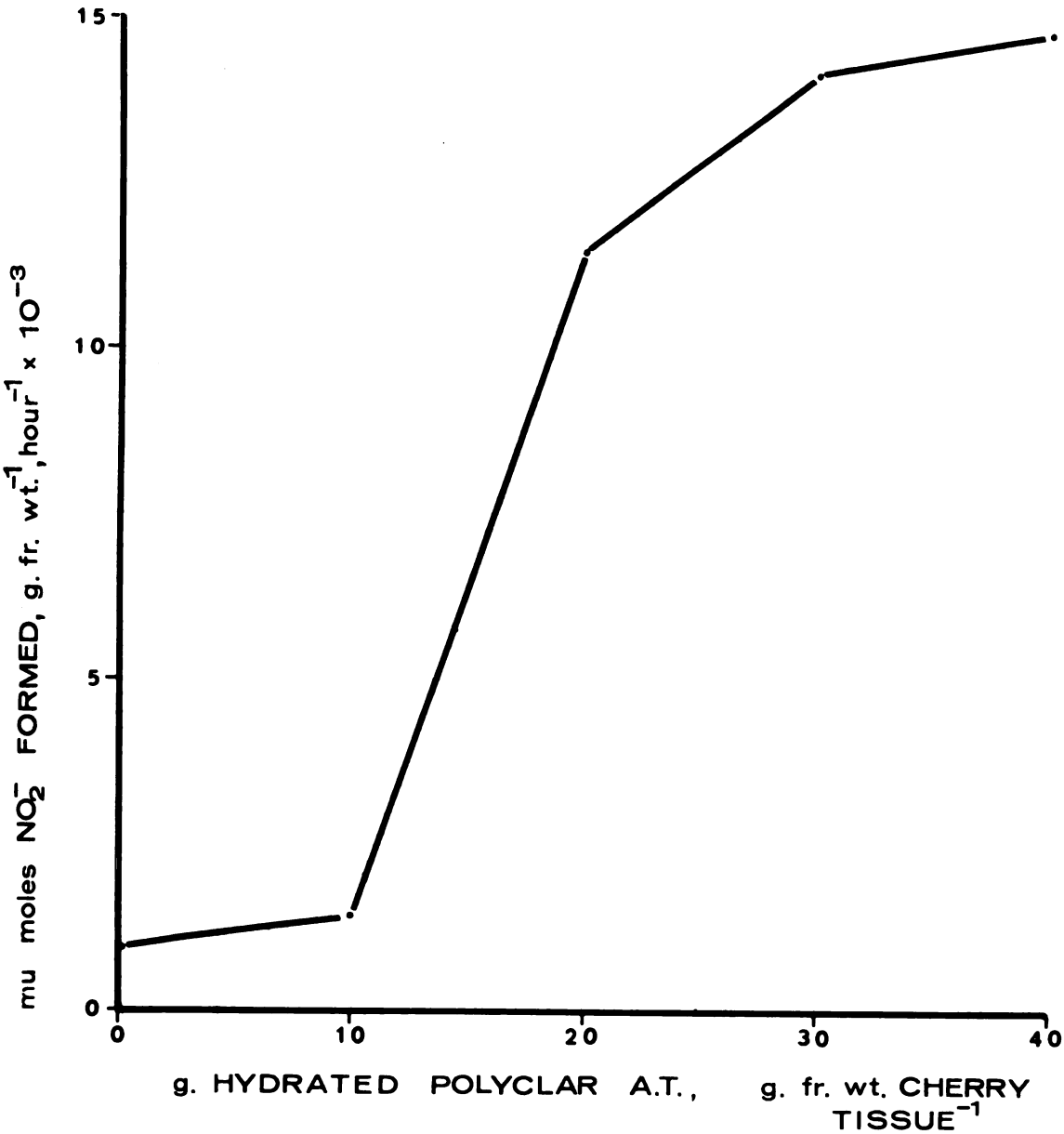
[x]0.5 g. oats leaf tissue was extracted with 10 g. hydrated PVP. either alone or in the presence of 0.5 g. fruit tree leaf tissue. Extraction medium comprised 50mM K_2HPO_4; 5mM EDTA; 10mM cysteine; pH 8.8.

[y]Data were statistically analysed following logarithmic transformation. Means followed by unlike letters differ significantly ($P < 0.01$).

[z]Apple, apricot, sour cherry and plum tissue contained a low level of enzyme activity which is expressed here as a percentage of the activity of the oats tissue.

Figure 8.--Effect of increasing amounts of hydrated insoluble polyvinylpyrrolidone (Polyclar A.T.) on the extraction of nitrate reductase from 0.5 g. oats leaf tissue in the presence of 0.5 g. sweet cherry leaf tissue.

Each point is the mean of 3 replications. F value for treatments was significant ($P < 0.01$).


15
10
5
0
0
10
20
30
40
mu moles NO_2^- FORMED, g. fr. $wt.^{-1}$, $hour^{-1}$ x 10^{-3}
g. HYDRATED POLYCLAR A.T., g. fr. wt. CHERRY TISSUE^{-1}

it was clear that at least 20 g. PVP. were needed per g. fresh weight cherry tissue. However it was at this ratio that the 38 per cent inhibition of the oats enzyme had occurred. In the studies with peach tissue, on the other hand, up to 60 g. PVP. per g. peach tissue produced negligible improvement in the amount of oats enzyme recovered (Table 6).

The first additional reductant added to the extraction medium was mercaptobenzothiazole. At concentrations up to 10mM it gave no improvement with sweet cherry tissue or peach tissue (Table 7). A more powerful reductant, dithiothreitol (Cleland's Reagent), was then added to the extraction medium. However at concentrations from 10^{-5} M to 10^{-2} M it gave no protection to the oats enzyme extracted in the presence of peach tissue (Table 8).

Nitrate Reductase in Leaves of Mature, Field-Grown Trees.

The petiole uptake technique was used to study the time-course for the induction of nitrate reductase in leaves from mature, field-grown apricot and sour cherry trees (Figures 9 and 10). Mid-shoot leaves from current season terminal shoots were selected for these studies. In apricot (Figure 9), the induction pattern corresponded closely to that previously found for sand-cultured trees (Figure 2). Interestingly, the field-grown leaves initially had a relatively high enzyme level and the

Table 6.--Effect of increasing amounts of hydrated insoluble polyvinylpyrrolidone (Polyclar A.T.) on the extraction of nitrate reductase from 0.5 g. oats leaf tissue in the presence of 0.5 g. peach leaf tissue.[x]

Treatment: Tissue	Treatment: PVP (g/g fresh wt. Peach tissue)	Nitrate reductase activity ($m\mu$ moles NO_2^- formed, g. fresh wt.$^{-1}$, hr.$^{-1}$)	
Oats	0	4768	a
Oats + peach	0	0	b
Oats + peach	20	18	b
Oats + peach	40	104	b
Oats + peach	60	139	b

[x]Extraction medium comprised 50 mM K_2HPO_4; 5mM EDTA; 10mM cysteine; and the appropriate amount of PVP.; pH 8.8. Means followed by unlike letters differ significantly ($P < 0.01$).

Table 7.--Effect of mercaptobenzothiazole in the extraction medium on the extraction of nitrate reductase from 0.5 g. oats leaf tissue in the presence of either 0.5 g. sweet cherry or 0.5 g. peach leaf tissue.[x]

Concentration of mercaptobenzothiazole	Nitrate reductase activity: Cherry	Nitrate reductase activity: Peach
(mM)	($m\mu$ moles NO_2^- formed, g. fresh wt.$^{-1}$, hour^{-1})	
0	13,106	338
1	14,042	450
10	13,652	700

[x]Extraction medium comprised: 10 or 15 g. hydrated polyvinylpyrrolidone per g. fresh weight for cherry or peach respectively plus 50mM K_2HPO_4; 5mM EDTA; 10mM cysteine; and the mercaptobenzothiazole as appropriate; pH 8.8. Means in each column do not differ significantly ($P < 0.05$).

Table 8.--Effect of dithiothreitol in the extraction medium on the extraction of nitrate reductase from 0.5 g. oats leaf tissue in the presence of 0.5 g. peach leaf tissue.[x]

Treatment		Nitrate reductase activity
Tissue	Dithiothreitol	
	(M)	(mμ moles NO_2^- formed, $g.\ fresh\ weight^{-1}$, $hour^{-1}$)
Oats	0	5583 a
Oats + peach	0	0 b
Oats + peach	10^{-5}	0 b
Oats + peach	10^{-4}	0 b
Oats + peach	10^{-3}	34 b
Oats + peach	10^{-2}	25 b

[x]Extraction medium comprised 10 g. hydrated polyvinylpyrrolidone per g. fresh weight; 50mM K_2HPO_4; 5mM EDTA; 10mM cysteine; and the dithiothreitol as appropriate; pH 8.8. Means followed by unlike letters differ significantly ($P < 0.01$).

Figure 9.--Time-course for the induction of nitrate reductase in leaves from mature, field-grown, apricot trees.

Excised mid-shoot leaves were incubated with their petioles immersed in either 10mM KNO_3 or 10mM KCl (control) for different periods up to 9 hours. Each point is the mean of 2 replications. F value for treatments was significant ($P < 0.01$).

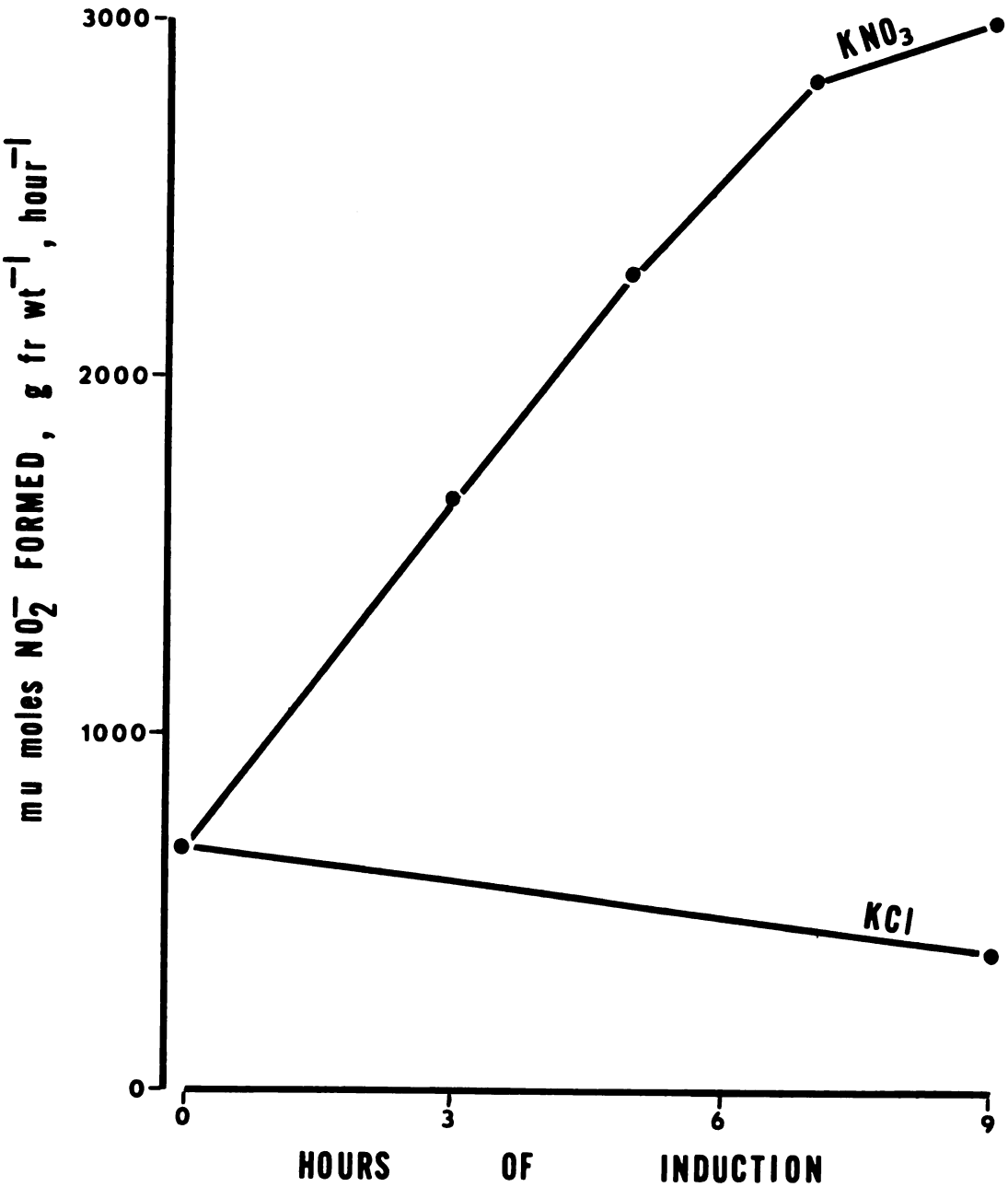
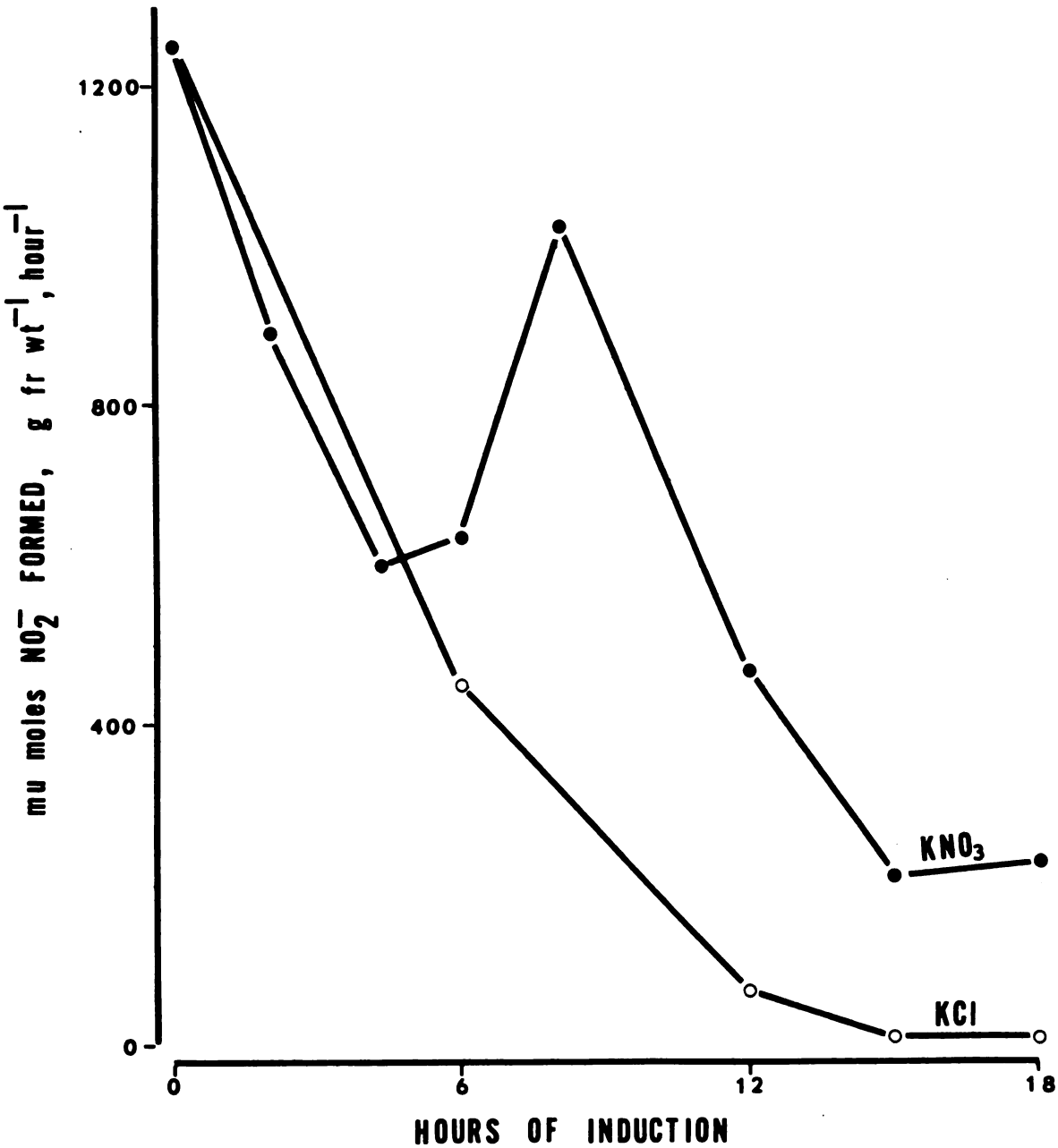

3000
2000
1000
0
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
KNO_3
KCl
0
3
6
9
HOURS OF INDUCTION

Figure 10.--Time-course for the induction of nitrate reductase in leaves from mature, field-grown, sour cherry trees.

Excised mid-shoot leaves were incubated with their petioles immersed in either 10mM KNO_3 or 10mM KCl (control) for different periods up to 18 hours. Each point is the mean of 2 replications. F value for treatments was significant ($P < 0.05$).


1200
800
400
0
mu moles NO_2^- FORMED, g fr wt^{-1}, $hour^{-1}$
KNO_3
KCl
0
6
12
18
HOURS OF INDUCTION

maximum enzyme activity attained was greater than that for the sand-cultured leaves. Sour cherry leaves (Figure 10) also showed enzyme induction after 8 hours. However the extremely high initial enzyme levels overshadowed the induction phenomenon.

Attempts to demonstrate enzyme induction by the petiole uptake technique in sweet cherry and plum leaves were not successful. Salt accumulated along the mid-rib and this indicated that translocation of the induction solution from the mid-rib region throughout the leaf blade was minimal. To some extent this was true also of sour cherry but not of apricot leaves.

Although enzyme induction was not demonstrated, enzyme activity was detected in both sweet cherry and plum leaves during these studies. Accordingly both the nitrate reductase activity and nitrate concentration of freshly collected field-grown leaves was determined for all four species (Table 9). Both enzyme and nitrate levels were lower than that produced in the sand-cultured trees by 15mM NO_3^- solution (Table 1), nevertheless sour cherry, sweet cherry and plum leaves contained detectable levels of nitrate and some sour cherry and plum leaves contained detectable levels of nitrate reductase. The trace of nitrate reductase reported in sweet cherry leaves (33 mμ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1}) was negligible.

Table 9.--Nitrate reductase activity and nitrate concentration of mid-shoot leaves of mature, field-grown stone fruit trees.[x]

Species	Nitrate reductase activity	Nitrate concentration
	($m\mu$ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1})	($m\mu$ moles, g. fresh weight^{-1})
Apricot	367	85
Sour cherry	150	204
Sweet cherry . . .	33	188
Plum	111	159

[x]Enzyme was extracted in presence of 20 g. hydrated PVP. per g. fresh weight of leaf tissue except for sweet cherry tissue which required 30 g. per g. to prevent the extract from turning brown. Extraction medium comprised 50mM K_2HPO_4; 5mM EDTA; 10mM cysteine; pH 8.8. Nitrate reductase was assayed using NADH as electron donor. Each value is the mean of 3 replications. Each sample comprised 5 mid-shoot leaves taken at shoulder height uniformly from around the periphery of each of 10 trees.

Discussion

Nitrate Reductase in Leaves of Young Trees.

Nitrate reductase was found in apricot, sour cherry, sweet cherry and plum leaves following the addition of excess nitrate ions to the root medium. The nitrate and enzyme levels in sour cherry, sweet cherry and plum leaves corresponded closely to those in the apple control but the enzyme levels were ca. half those previously reported for apple seedling leaves (Klepper and Hageman 1969) but were similar to those reported following induction in leaves from mature apple trees. Apricot leaves attained three times the levels that the other species attained. In part this may reflect the difficulty in extracting an active enzyme from the other tissues particularly sour cherry and sweet cherry.

Classically nitrate reductase has been regarded as substrate (NO_3^-) inducible and Klepper and Hageman (1969) noted that the level of nitrate in the tissue appears to be a major factor limiting the level of nitrate reductase. Nevertheless in the studies reported herein it was found with apricot trees that nitrate reductase was negatively correlated with nitrate concentration, yet addition of nitrate to the root medium increased leaf enzyme activity.

Apparently the apricot leaves responded to the increase in nitrate concentration by synthesising nitrate

reductase to such an extent that nitrate accumulation beyond a threshold level did not occur. Apparently apple tissue was much slower to adjust to the increase in leaf nitrate concentration and thus permitted nitrate to accumulate, though, as the petiole induction studies showed, nitrate reductase was induced by nitrate.

Relatively high levels of nitrate reductase (ca. 500 mμ moles NO_2^- formed, g. fresh weight^{-1}, hour^{-1}) were found in apricot and apple leaves in the absence of applied nitrate. Microbial oxidation of the NH_4^+ in the root medium (the medium was not sterile) to NO_3^- may have been responsible, in part at least, for this activity. Similar levels were found in field-grown apricot leaves in this study and in field-grown apple leaves by Klepper and Hageman (1969). Whether the enzyme is ever completely absent in these tissues is not known. It would seem, however, that trace amounts of nitrate may be transported to the leaves in the absence of excess nitrate in the root medium and that this is sufficient to maintain a threshold level of nitrate reductase in the tissue.

In this regard, the time course studies were interesting. When Filner (1966) conducted a similar study using cultured tobacco cells he found a 6-hour lag period occurred prior to enzyme synthesis, which implied that at the beginning of the experiment the cells contained neither a permease system for nitrate uptake nor any nitrate

reductase. The absence of a lag period, both in the present study and in that of Klepper and Hageman (1969), indicated that prior to the experiment the leaves had received nitrate and that both an active nitrate permease system and nitrate reductase were present.

The time-course and substrate concentration studies confirmed that the enzyme was substrate inducible. Interestingly, the optimum nitrate concentration for enzyme activity (ca. 10mM NO_3^-) corresponded to that of standard nutrient solutions e.g. 12mM NO_3^- of the Long Ashton solution (Hewitt 1966).

While NADH is normally regarded as the electron donor for nitrate reductase (Beevers and Hageman 1969), the enzyme most commonly found in plant leaves is capable of accepting electrons directly from either NADH or $FMNH_2$ (but only indirectly from NADPH[1]). In the present study it was demonstrated that apricot leaf nitrate reductase could also use both NADH and $FMNH_2$ as electron donors, but $FMNH_2$ was less than half as effective as NADH. This also held true for the oats enzyme and an admixture of apricot tissue with the oats tissue during extraction did not reduce the $FMNH_2$ activity of the oats. It is concluded that either apricot nitrate reductase cannot use $FMNH_2$ as

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[1]Reduced nicotinamide adenine dinucleotide phosphate.

effectively as NADH or that the differences in activity were due to the inadequacy of the $FMNH_2$ assay technique. The latter explanation is favored in view of the results with the oats enzyme. It should be noted that the $FMNH_2$ activity reported for apple leaf nitrate reductase by Klepper and Hageman (1969) was only 54 per cent of the NADH activity.

To summarise the studies with young trees, nitrate was found in the leaves of apple, apricot, sour cherry, sweet cherry, peach, and plum trees after excess nitrate had been applied to the root medium. An NADH-dependent nitrate reductase was, at the same time, extracted from the leaves of all species except peach. The apricot enzyme was two to three times as active as the enzymes from the other species including apple and was shown to be substrate (NO_3^-) inducible and to be able to use either NADH or $FMNH_2$ as its electron donor. It would thus seem to be typical of nitrate reductase as commonly found in plant leaves, as described by Beevers and Hageman (1969).

The Extraction Procedure.--Inclusion of PVP. in the extraction medium protected apricot nitrate reductase from inactivation during extraction and partially purified the enzyme extract. More PVP. was found necessary than that reported for apple tissue by Klepper and Hageman (1969) but the extra PVP. was associated with greater purification of the enzyme. Oats tissue responded similarly to apricot.

However PVP. failed to prevent the complete inactivation of the oats enzyme when extracted in the presence of peach tissue or its partial inactivation when extracted with sour cherry or sweet cherry tissue. Although this partial inactivation was not shown to be statistically significant it should nevertheless be considered as a real effect. The nitrate reductase assay procedure was only semi-quantitative and large differences were needed for significance. Then the high values for oats plus apple, apricot and plum respectively probably masked the partial inactivation. Subsequent studies with mid-shoot leaves taken from mature trees in mid-summer (data not presented) showed almost complete inactivation of oats nitrate reductase extracted in the presence of sour cherry and sweet cherry tissue. The inactivation of oats nitrate reductase by peach tissue also suggests that the failure to find nitrate reductase in peach leaves may have been due to inactivation of the enzyme during extraction rather than to the non-occurrence of the enzyme in the tissue.

The protection of enzymes during extraction from plant tissue has been reviewed by Loomis and Battaile (1966) and Anderson (1968). It has proved particularly difficult to isolate enzymes from fruit tree tissues because of the high phenolic content of such tissues. In living cells, prior to senescence, phenolic compounds are frequently associated with vacuoles and are thus spacially

separated from cytoplasmic enzymes. However, during enzyme extraction, tissue homogenization destroys this spacial separation and phenolic compound - protein interactions may occur.

Phenolic compounds may inactivate proteins in two ways (Loomis and Battaile 1966). First the phenol may form reversible hydrogen bonds with proteins. The phenol H-bond to N-substituted amides is one of the strongest types of H-bond. Nylon or PVP. have such a reactive center and H-bond to phenols in a manner similar to protein. Secondly phenols are readily oxidised to quinones both non-enzymatically and by cytoplasmic enzymes (e.g. phenol oxidases and peroxidases). This is the browning reaction typical of senescing tissue. Quinones are oxidizing agents and may oxidize essential groups of proteins (e.g. -SH groups). More important, they polymerise rapidly, and in the presence of protein they also react rapidly to form covalent bonds to the protein. This second type of phenol-protein interaction is irreversible.

Thus techniques for isolating enzymes from tissues high in phenolic compounds should specifically separate the phenols from the proteins, and at the same time prevent oxidation of the phenols. Phenols which form H-bonded complexes with proteins (condensed tannins) are effectively removed by adding large amounts of substances which contain groups similar to the peptide linkage (e.g. PVP.). However

there are some phenolic compounds *viz*. "hydrolyzable tannins" (*e.g.* catechol [o-diphenol] derivatives) which, as a result of internal H-bonding, do not form strong H-bonded complexes with proteins or PVP., yet are readily oxidized to quinones. PVP. would not effectively remove such compounds and they would remain in the extract as latent inhibitors.

In the present study efforts to protect the sweet cherry and peach enzymes were initially directed towards use of additional PVP. In both instances 20 g. PVP. per g. fresh weight had effectively inhibited the browning reaction yet enzyme inactivation was obviously occurring. It was postulated that oxidation of phenols had been prevented but that phenol-nitrate reductase H-bonding was still occurring. However when additional PVP. failed to increase enzyme activity, this hypothesis was rejected. It was then considered that hydrolyzable tannins of low molecular weight which were not removed by PVP. might be responsible for the inactivation, which would have occurred subsequent to their oxidation. Anderson (1968) reported that reducing agents had proved particularly effective for increasing enzyme extraction efficiency from tissues containing o-diphenoloxidase substrates of low molecular weight. Thus strong reducing agents were added to the extraction medium to counter these compounds. However neither mercaptobenzothiazole, which is a powerful inhibitor of o-diphenoloxidase

(Anderson 1968), nor dithiothreitol at concentrations up to 10mM improved enzyme activity. It should also be noted that 10mM cysteine, which is also a reducing agent and an inhibitor of o-diphenoloxidase, had routinely been included in the extraction medium.

It is presently considered that the hydrolyzable tannins hypothesis deserves further testing. Reducing agents operate (Loomis and Battaile 1966) not by preventing the oxidation of phenols, but rather by rapidly removing the quinone that is formed, thus preventing quinone accumulation and reducing the probability of quinone-protein reactions. Loomis and Battaile (1966) suggested that gel filtration or dialysis might be more effective in removing these materials than relying on reducing agents. They suggested poly-N-methylacrylamide would very likely combine a capacity for gel filtration with a capacity to absorb phenols. The problem with this technique would be preventing oxidation prior to and during filtration. In addition to using reducing agents, grinding the tissue in liquid nitrogen might assist in this regard.

Returning to the PVP. experiments, the amount of PVP. found necessary even for apricot tissue (10 to 20 g. per g. fresh weight) seems rather high when compared to the 1.5 g. per g. fresh weight found necessary by Loomis and Battaile (1966) for peppermint. It is possible that the pH of the extraction media in the present study was

too high. The extractant pH of 8.8 was selected firstly because it was used successfully by Klepper and Hageman (1969) in isolating nitrate reductase from apple leaves, and secondly because it worked well in preliminary studies with apricot leaves in the present investigations.

However the optimum pH for absorption of phenolics by PVP. is 3.5 (Loomis and Battaile 1966; Anderson 1968). Hydrolyzable tannins are bound very strongly at pH 3 to 4 and binding decreases markedly from pH 5 to 7.5. However condensed tannins are bound almost independently of pH below 7 to 8. The binding decreases rapidly above pH 8. Loomis and Battaile (1966) point out that above pH 7.5 the phenols become increasingly ionised. The ionised phenols cannot H-bond with either protein or PVP., and at the same time they are more readily oxided than non-ionised forms.

In the present studies the initial pH of the extraction medium was 8.8 and the pH of the supernatant (crude enzyme extract) ranged from 8.0 (for oats 0.5 g. + peach 0.5 g.) to 8.4 (oats 0.5 g.). Thus there would seem merit in testing nitrate reductase extraction at pHs from 6.0 to 7.5. This may reduce the amount of PVP. required and improve enzyme recovery.

To summarise the studies of the extraction procedure, PVP. made possible the extraction of an active nitrate reductase from apricot, sour cherry, sweet cherry and plum leaves. However, it provided only partial

protection to sour cherry and sweet cherry. The failure to find nitrate reductase in peach tissue was probably due to inability to protect the enzyme during extraction. It is proposed that lowering the extraction pH to 6.0 to 7.5 to favor phenol absorption by PVP., grinding the tissue in liquid nitrogen to keep it cold and anaerobic, and submitting the supernatant to gel filtration to remove hydrolyzable tannins of low molecular weight prior to their oxidation, may improve enzyme extraction from sour cherry and sweet cherry and permit enzyme extraction from peach leaves.

The results of the present studies further indicate that apricot and plum tissues may contain low quantities of hydrolyzable tannins, that sour cherry and sweet cherry tissues may contain intermediate quantities, and that peach tissue may contain high quantities.

Nitrate Reductase in Leaves of Mature, Field-Grown Trees.

Nitrate ions were detected in mid-shoot leaves from field-grown apricot, sour cherry, sweet cherry, and plum trees and nitrate reductase was detected in apricot leaves and in a few samples of sour cherry and plum leaves. The levels found were low but convincing, the levels in apricot corresponding to those previously reported for apple (Klepper and Hageman 1969). Clearly the application of excess nitrate fertilizer to the root system was not essential for a small quantity of nitrate to be translocated to the leaves and hence induce nitrate reductase activity.

The induction studies with excised apricot and sour cherry leaves showed that field-grown leaves of these species can achieve enzyme activities comparable to greenhouse-grown leaves. The high initial enzyme level found with sour cherry was surprising and subsequent samples had much lower levels. A similar induction pattern with a peak between 7 to 9 hours was obtained when the experiment was repeated with leaves with a low initial enzyme level.

The finding of nitrate reductase in field-grown apricot, sour cherry, sweet cherry and plum leaves, together with its induction in apricot and sour cherry leaves, indicates that leaves of Prunus spp. can metabolise nitrate and that these species should be able to utilize nitrate foliar sprays provided nitrate ions can enter the leaves through the cuticle.

SAND CULTURE AND FIELD EVALUATIONS OF NITRATE FOLIAR SPRAYS FOR PRUNUS SPP.

In the preceding section it was demonstrated that Prunus leaves could metabolise nitrate ions. Thus it would be expected that stone fruit trees could utilize nitrate foliar sprays beneficially, provided, of course, that sufficient nitrate could be absorbed by the leaves to influence tree growth and fruit development. The Review of Literature recorded very little information relating to the response of stone fruit trees to nitrate foliar sprays, the information available suggesting nil responses would be obtained under commercial conditions.

In order to better establish the response of stone fruit trees to nitrate foliar sprays evaluations of such sprays were undertaken in sand culture and field experiments. The basic evaluation criterion used was leaf nitrogen concentration, for unless the foliar sprays could increase the leaf nitrogen concentration of nitrogen deficient trees in a manner comparable to soil nitrogen applications, commercially more meaningful criteria such as tree growth and fruit yield would not be affected by the sprays.

Materials and Methods

Sand Culture Experiments

Two experiments were conducted on young trees growing in sand culture. In the first, different cations were evaluated as carriers for nitrate ions in foliar sprays when applied to peach and sweet cherry trees. In the second, the absorption and translocation of 4 per cent potassium nitrate foliar sprays was assessed in young apricot trees.

Plant Material.--The trees used in these experiments were 4-year-old sweet cherry trees (Prunus avium L. cv. "Windsor"), 2-year-old peach trees (Prunus persica [L.] Batsch. cv. "Glohaven"), and 1-year-old apricot trees (Prunus armeniaca L. cv. "Goldcot"). The trees were grown either in calcined clay ("Turface": Wyandotte Chemicals Corp., Wyandotte, Mich.) (expt. 1) or sand (expt. 2) and were maintained in moderate vigor and low nitrogen status with the modified Long Ashton solution (Hewitt 1966) described earlier (p. 29) of which each tree received 2 litres twice per week. Any additional water requirement was supplied as deionised water. When it was desired to bring the trees into nitrogen deficiency i.e. immediately prior to and during an experiment, the $(NH_4)_2SO_4$ was omitted from the nutrient solution.

Prior to the commencement of each experiment the trees were brought in a dormant condition from cold storage

(38° F) where they had been kept in darkness with occasional watering to prevent drying out, for at least 10 weeks. The carrier ion experiment was conducted out-of-doors in mid-summer and the absorption experiment was conducted in a greenhouse during spring with a 16-hour photoperiod of supplemental light (200 ft. candles, cool-white fluorescent, at leaf level). Environmental conditions and insect control procedures were similar to those described in the preceding section. In the first experiment the trees were pruned to three shoots and in the second experiment to two shoots.

Treatments and Experimental Design.--In the carrier ion experiment treatments were foliar sprays of nitrogen at 0.2 g. equiv. per litre (being equivalent in nitrogen to a 2 per cent KNO_3 spray) as: NH_4NO_3 0.1M; KNO_3 0.2M; $NaNO_3$ 0.2M; $Ca(NO_3)_2$ 0.1M; $Mg(NO_3)_2$ 0.1M (sweet cherry only); plus a 0.1M urea control and an untreated control. The treatments were applied four times to the cherry trees and six times to the peach trees at weekly intervals. A completely randomised design with three replications and single tree plots was employed.

In the absorption experiment a 0.4M (i.e. 4 per cent) KNO_3 foliar spray was compared with a soil application of 15mM NO_3^- (supplied by replacing the 2.5mM K_2SO_4 and 5mM $CaCl_2$ of the nutrient solution with 5mM KNO_3 and 5mM Ca $(NO_3)_2$) and an untreated control. Three applications were made at weekly intervals the first application

once the fifth leaf was fully expanded. A randomised block design with 3 replications and single tree plots was employed.

In both experiments 0.1 per cent X77 surfactant (containing alkylarylpolyoxyethylene glycols, free fatty acids, and isopropanol: Chevron Chemical Co., Ortho Division, San Francisco, Calif.) was included in each spray. The sprays were applied on each occasion until spray dripped from the leaves and the sand medium was protected against spray drip during treatment by plastic sheeting.

Leaf Analyses and Growth Measurements.--In the carrier ion experiment, one week after the last spray application leaf injury was rated visually on a 0-10 scale of increasing severity and leaf samples were collected for nitrogen, potassium, sodium, calcium and magnesium analysis. Heavy rain two days before sampling rendered leaf washing unnecessary.

In the absorption experiment, samples of sprayed leaves were collected for nitrate reductase assay 24 hours after each spray application. Three weeks after the last spray application, the length, dry weight, and nitrogen and potassium content of the new, unsprayed, shoot growth were measured.

Leaf samples for nutrient analysis were dried at 65° C for 24 hours, milled to 20 mesh fineness, then re-dried at 105° C for 1 hour prior to sub-sampling. Nitrogen

was determined by a macro-Kjeldahl procedure employing Gunning's catalyst (Lepper 1945). Potassium was analysed on a water extract (0.5 g. leaf powder per 100 ml. water) by flame emission spectroscopy using a Beckman Model B spectrophotometer with flame attachment. Sodium, calcium, and magnesium were analysed on a nitric acid solution of plant ash (0.5 g. leaf powder dry ashed at 500° C for 8 hours; ash dissolved in 5 ml. of 2 N HNO_3) by spark emission spectroscopy using an Applied Research Laboratories "Quantograph". Nitrate reductase was extracted from fresh leaf tissue by the method of Klepper and Hageman (1969) and assayed by the method of Hageman and Flesher (1960).

Field Experiments

Autumn and spring KNO_3 sprays were evaluated as nitrogen sources for peach trees in two field experiments, the first being conducted in the 1968-69 season and the second in the 1969-70 season. Each experiment was conducted in a separate commercial block[1] of freestone peach trees (Prunus persica [L.] Batsch. cv. "Richaven") which at the beginning of the experiment was 4-years old and had produced one commercial crop. Leaf analysis data and visual leaf symptoms indicated that each block was moderately nitrogen deficient.

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[1]Orchard of Mr. H. Rapp, 63545 S. Van Dyke Rd., Romeo, Michigan.

Treatments and Experimental Design.--The treatments applied in experiment 1 were:

(i) Soil nitrogen (a) 0.5 lb. nitrogen per tree in autumn (October 1968).
(b) Control - no soil nitrogen.

(ii) Foliar nitrogen: 1.8 per cent (15 lb. per 100 gal.) KNO_3, fertilizer grade.
(a) three post-harvest sprays at five day intervals commencing on October 5, 1968.
(b) three spring sprays at five day intervals commencing with the first cover spray (May 31, 1969).
(c) control - unsprayed.

A split plot design was employed with soil nitrogen treatments as the whole plots and spray treatments as the sub plots. There were four replications. Single tree sub plots without internal guard rows were used. Internal guard rows were considered unnecessary as tree spacing was 24 feet by 30 feet and sprays were not applied under windy conditions.

The treatments in experiment 2 were:

(i) Untreated control

(ii) Soil nitrogen: 0.5 lb. nitrogen per tree in autumn (October 1969).

(iii) Foliar nitrogen: three post-harvest sprays plus three spring sprays, containing 0.4 g. equiv. nitrogen per litre, were applied at five day intervals as:
(a) 1.2 per cent (10 lb. per 100 gal.) urea, fertilizer grade (2.5 per cent biuret).
(b) 4.0 per cent (33.7 lb. per 100 gal.) KNO_3, fertilizer grade.
The first post-harvest spray was applied on September 29, 1969, and the first spring spray on May 26, 1970.

A randomised block design with four replications and two-tree plots was employed. Each plot was surrounded by a single guard row.

In the soil nitrogen treatments, the nitrogen was applied as ammonium nitrate, fertilizer grade, and was hand broadcast around the periphery of the tree. All foliar sprays contained 0.03 per cent sodium dioctyl sulphosuccinate ("Sur Ten": American Cyanamid Corp., 70 per cent active ingredient) surfactant, and were applied with a high pressure single nozzle gun until spray dripped from the leaves (ca. 2 gallons per tree).

Leaf Analyses and Growth Measurements.--A leaf sample was taken for nitrogen and potassium analysis from each tree where appropriate (a) five days after the last autumn spray application (b) unsprayed spring leaves (c) mature leaves in mid-summer (July). Each sample consisted of 40 mid-shoot leaves taken uniformly from around the periphery at shoulder height. Leaves were not washed because in each instance where the leaves sampled had been sprayed, heavy rain had fallen between the last spray application and leaf sampling. Sample preparation and nutrient analyses were by the methods used in the sand culture experiments.

The influence of autumn treatments on late autumn tree growth was determined by measuring terminal shoot growth during the winter immediately following treatment.

The influence of experiment 1 treatments on tree growth during the summer of treatment was assessed by measuring trunk circumference at 18 inches above the ground and terminal shoot growth during the following winter.

Statistical Analyses.

All data were analysed by standard statistical procedures (Steel and Torrie 1960) and treatment means were compared by Tukey's ω-procedure.

Results

Sand Culture Experiments

Evaluation of Cations as Carriers for Nitrate Foliar Sprays.--The first sand culture experiment compared NH_4^+, K^+, Na^+, Ca^{+2}, and Mg^{+2} (sweet cherry only) as carriers for nitrate foliar sprays at the same nitrogen concentration *viz*. 0.2 g. equiv. per litre, which is equivalent in nitrogen to a 2 per cent KNO_3 spray. Peach leaves were found to be much less susceptible to injury by sprays at this concentration than were sweet cherry leaves (Tables 10 and 11). Severe injury to cherry leaves and moderate injury to peach leaves was caused by Na^+ and Ca^{+2}. The injury on sweet cherry leaves was a severe marginal necrosis and leaf roll characteristic of salt toxicity. Symptoms were well established after the second spray application. The injury on peach leaves consisted of tip necrosis and

Table 10.--Effect of nitrogen foliar sprays at 0.2 g. equiv. per litre on leaf injury[x] and leaf nutrient concentration[y] as affected by carrier form[z]. A. Sweet cherry trees.

Carrier form	Injury	Leaf nutrient concentration				
		N	K	Na	Ca	Mg
		(%)	(%)	(%)	(%)	(%)
Controls:						
unsprayed	0	1.77 a	1.38 b	0.017 a	1.20 a	0.41 bc
urea spray	2	2.21 b	1.27 b	0.010 a	1.06 a	0.23 a
Nitrate sprays:						
NH_4^+	2	1.94 ab	1.31 b	0.014 a	1.24 a	0.37 b
K^+	1	1.84 a	2.00 c	0.018 a	0.96 a	0.33 ab
Na^+	9	2.01 ab	1.01 ab	0.392 b	1.07 a	0.32 ab
Ca^{+2}	7	2.09 ab	1.09 ab	0.044 a	2.24 b	0.32 ab
Mg^{+2}	4	1.91 ab	0.84 a	0.009 a	1.05 a	0.52 c

[x]Injury rated on a 0-10 scale of increasing severity.

[y]Concentration expressed on a dry weight basis.

[z]Means in each column followed by unlike letters differ significantly ($P < 0.05$).

Table 11.--Effect of nitrogen foliar sprays at 0.2 g. equiv. per litre on leaf injury[x] and leaf nutrient concentration[y] as affected by carrier form[z]. B. Peach trees.

Carrier form	Injury	Leaf nutrient concentration			
		N	K	Na	Ca
		(%)	(%)	(%)	(%)
Controls:					
unsprayed	0	3.21 a	1.95 a	0.010 a	1.30 ab
urea spray	0	3.17 a	1.78 a	0.011 a	1.20 a
Nitrate sprays:					
NH_4^+	0	3.09 a	1.79 a	0.018 a	1.25 ab
K^+	0	3.50 a	2.09 a	0.017 a	1.26 ab
Na^+	3	3.56 a	1.92 a	0.142 b	1.11 a
Ca^{+2}	3	3.57 a	1.99 a	0.015 a	1.79 b

[x]Injury rated on a 0-10 scale of increasing severity.

[y]Concentration expressed on a dry weight basis.

[z]Means in each column followed by unlike letters differ significantly ($P < 0.05$).

some marginal necrosis. The slight injury caused by K^+, NH_4^+ and the urea spray control on sweet cherry leaves was principally a tip necrosis which appeared to be caused by external spray residues being higher at the leaf tip.

The nitrate sprays, regardless of carrier ion, failed to significantly increase the leaf nitrogen concentration of either the peach or sweet cherry trees (Tables 10 and 11). In contrast, the urea spray control increased sweet cherry leaf nitrogen concentration.

However the carrier ions tended to raise the concentration of their respective elements in the sprayed leaves. In sweet cherry, leaf potassium, sodium, calcium, and magnesium were increased by K^+, Na^+, Ca^{+2}, and Mg^{+2} sprays respectively, although the Mg^{+2} spray did not increase leaf magnesium significantly above the unsprayed control value. Also, apparent urea x magnesium and potassium x magnesium antagonisms were found, both the urea and K^+ sprays decreasing leaf magnesium. In peach, the Na^+ spray increased leaf sodium and the Ca^{+2} spray tended to increase leaf calcium (though not significantly above the unsprayed control value), however the K^+ spray did not increase leaf potassium.

Absorption and Translocation of Potassium Nitrate Foliar Sprays.--When 4 per cent KNO_3 foliar sprays were applied at weekly intervals for 3 weeks to the leaves of 1-year-old apricot trees in the greenhouse during the early

period of spring growth, leaf nitrate reductase activity was increased within 24 hours (Table 12). This indicates that nitrate absorption and initial metabolism occurred in the leaves. However the final spray application caused extensive tip and marginal leaf necrosis and reduced leaf photosynthetic area by ca. 50 per cent.

Table 12.--Effect of 4 per cent potassium nitrate foliar sprays on apricot leaf nitrate reductase activity and leaf injury.[x]

Treatment[y]	Nitrate reductase activity			Injury[z]
	Day 2	Day 9	Day 16	
	(mμ. moles NO_2^- formed, g. fr. wt.$^{-1}$, hr.$^{-1}$)			
Untreated control . . .	275 a	467 a	209 a	0
Soil nitrate control . .	292 a	1271 b	457 ab	0
Foliar nitrate	450 b	1300 b	975 b	5

[x]Means in each column followed by unlike letters differ significantly ($P < 0.05$).

[y]Treatments were applied on Days 1, 8, and 15.

[z]Injury rated on a 0-10 scale of increasing severity which occurred within 24 hours subsequent to the last spray application.

Terminal shoot growth made in the 3 weeks subsequent to the final spray application (Table 13) was stunted by the foliar nitrate treatments but the associated reductions in dry weight and shoot potassium content were not significant. Both the soil and foliar nitrate treatments produced a higher terminal shoot nitrogen concentration than the

Table 13.--Effect of 4 per cent potassium nitrate foliar sprays on growth[x] and nutrient content of apricot tree terminal shoots.[y]

Treatment	Length	Dry weight	Nitrogen		Potassium	
			concentration	content	concentration	content
	(cm)	(g)	(%)	(g)	(%)	(g)
Untreated control	16.4 a	3.50 a	1.28 a	0.046 a	4.06 a	0.148 a
Soil nitrate control	22.7 a	4.32 a	2.37 b	0.100 b	3.32 a	0.149 a
Foliar nitrate. .	7.4 b	2.02 a	2.12 b	0.043 a	3.97 a	0.080 a

[x]Growth made in the three weeks subsequent to the final spray application.

[y]Means in each column followed by unlike letters differ significantly ($P < 0.05$).

untreated control but only the soil nitrate treatment increased shoot nitrogen content. Thus, no evidence of nitrogen or potassium translocation from foliar nitrate treated leaves to their subtended terminal shoots was found.

Field Experiments

Experiment 1.--In the 1968-69 season the influence of an autumn soil nitrogen application on the nitrogen status of moderately nitrogen deficient commercial peach trees was compared with that of autumn and spring foliar sprays of KNO_3. An October soil application of 0.5 lb. nitrogen per tree (as 1.5 lb. of NH_4NO_3) increased the nitrogen concentration of both young spring leaves and mature leaves in mid-summer (Table 14). On the basis of Kenworthy's (1950) standards for peach trees in Michigan, this leaf nitrogen increase represented a change from a nitrogen shortage to a normal leaf nitrogen level. The soil nitrogen treatment also reduced the mid-summer leaf potassium concentration, however the concentration remained "normal".

Three post-harvest sprays of 1.8 per cent KNO_3 increased both the nitrogen and potassium concentrations of the sprayed leaves prior to leaf fall (Table 14) but had no effect on either the leaf nitrogen or potassium concentration of the following season's growth. There was,

Table 14.--Effect of certain soil and foliar applications of nitrogen on leaf nitrogen and potassium concentrations of commercial peach trees (1968-69 season).[x]

Treatment	Nitrogen			Potassium		
	Leaf fall	Spring	Mid-summer	Leaf fall	Spring	Mid-summer
	(%)	(%)	(%)	(%)	(%)	(%)
Soil nitrogen-autumn						
Treated	2.26 a	5.50 a	3.76 a	1.49 a	1.97 a	1.52 a
Untreated	2.20 a	5.06 b	2.96 b	1.60 a	2.25 a	1.89 b
Foliar nitrogen - autumn						
Treated	2.33 a	5.40 a	3.39 a	1.67 a	2.08 a	1.68 a
Untreated	2.14 b	5.16 a	3.33 a	1.42 b	2.14 a	1.73 a
Foliar nitrogen-spring						
Treated			3.46 a			1.78 a
Untreated			3.33 a			1.73 a

[x]Main effect means for each treatment set in each column followed by unlike letters differ significantly ($P < 0.05$).

however, a tendency for the post-harvest sprays to increase the leaf nitrogen concentration of the following season's growth of trees which had not received any soil nitrogen in the autumn (Figure 11a); however, this increase was not significant.

Three spring sprays of 1.8 per cent KNO_3 had no effect on either the nitrogen or potassium concentration of mid-summer leaves (Table 14). There was again, however, a non-significant tendency for the spray treatment to increase the nitrogen concentration of trees which had not received any soil nitrogen the previous autumn (Figure 11b).

Neither soil nor foliar post-harvest applications of nitrogen had any effect on terminal shoot growth prior to winter in the season (1968) of application (Table 15). The soil nitrogen treatment increased both terminal shoot growth and trunk circumference during the 1969 growing season; however, the foliar nitrogen treatments did not affect either.

Experiment 2.--In the 1969-70 season Experiment 1 was essentially repeated except that the foliar spray concentration was doubled, the spray x soil nitrogen interaction was not examined and a urea spray control was added. Additionally, the spring spray treatments were superimposed on the autumn spray treatments.

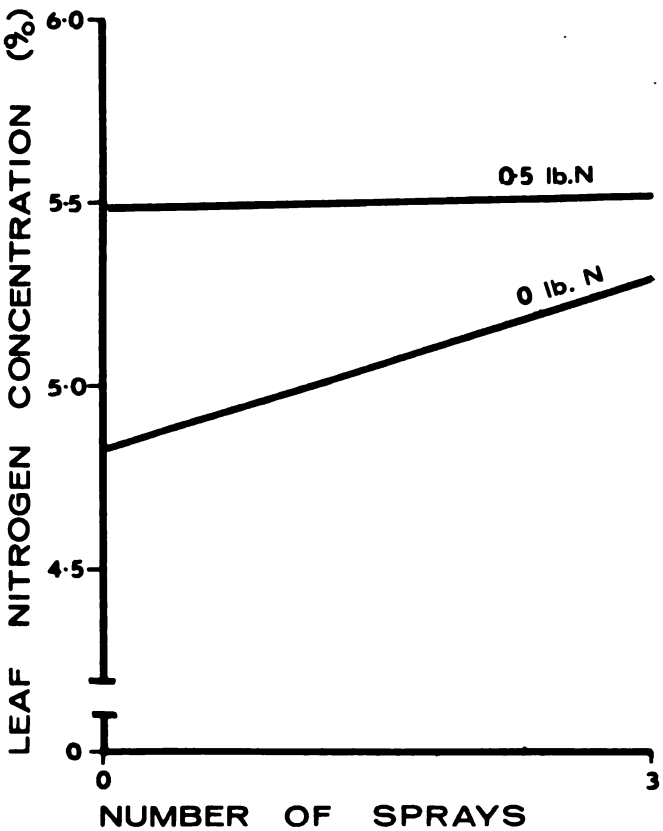
As in the 1968-69 season, an October soil application of 0.5 lb. nitrogen per tree increased the nitrogen

Figure 11.--Differential response of leaf nitrogen concentration to autumn and spring foliar sprays of 1.8 per cent potassium nitrate at different soil nitrogen levels (viz. 0 and 0.5 pounds nitrogen per tree).

(a) Response to autumn foliar sprays by mid-spring (Interaction F value not significant; ($P < 0.05$).

(b) Response to spring foliar sprays by mid-summer (Interaction F value not significant; ($P < 0.05$).

(a)


LEAF NITROGEN CONCENTRATION (%)
6·0
5·5
5·0
4·5
0
0·5 lb.N
0 lb. N
0
3
NUMBER OF SPRAYS

(b)

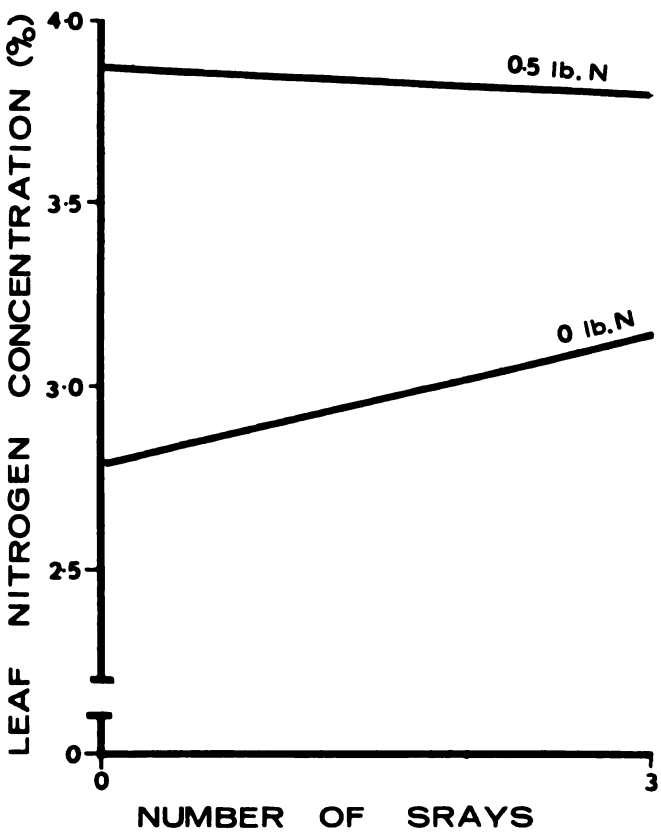

LEAF NITROGEN CONCENTRATION (%)
4·0
3·5
3·0
2·5
0
0·5 lb. N
0 lb.N
0
3
NUMBER OF SRAYS

Table 15.--Effect of certain soil and foliar applications of nitrogen on growth of commercial peach trees (1968-69 season).[x]

Treatment	Terminal shoot growth		Trunk circumference
	Winter 1968-69	Winter 1969-70	Winter 1969-70
	(inch)	(inch)	(inch)
Soil nitrogen - autumn			
Treated	18.4 a	20.6 a	9.40 a
Untreated	19.0 a	15.4 b	8.31 b
Foliar nitrogen - autumn			
Treated	18.7 a	18.2 a	8.87 a
Untreated	18.7 a	17.4 a	8.65 a
Foliar nitrogen - spring			
Treated		18.3 a	9.02 a
Untreated		17.4 a	8.65 a

[x]Main effect means for each treatment set in each column followed by unlike letters differ significantly ($P < 0.05$).

concentration of both young spring leaves and mature leaves in mid-summer (Table 16). Neither leaf potassium concentration, nor terminal shoot growth immediately following treatment, were affected by this treatment.

Three post-harvest sprays of 4 per cent KNO_3 again increased both the nitrogen and potassium concentrations of the sprayed leaves prior to leaf fall (Table 12), but had no effect on either the leaf nitrogen or potassium concentration of young spring leaves the following season. Similarly three post-harvest sprays of 1.2 per cent urea increased the nitrogen concentration of the sprayed leaves prior to leaf fall but had no effect on the nitrogen concentration of young spring leaves the following season. Further, neither set of post-harvest sprays had any effect on terminal shoot growth immediately following treatment.

The combination of three autumn plus three spring sprays either as 4 per cent KNO_3 or as 1.2 per cent urea failed to influence mid-summer leaf nitrogen or potassium concentrations (Table 16).

Discussion

Sand Culture Experiments

Carrier Ion Experiment.--The evaluation of cations as carriers for nitrate foliar sprays at the same nitrogen concentration revealed interesting differences in phytotoxicity. Salt injury (Ehlig and Bernstein 1959) may

Table 16.--Effect of certain soil and foliar applications of nitrogen on leaf nitrogen and potassium concentrations and terminal shoot growth of commercial peach trees (1969-70 season).[x]

Treatment	Nitrogen			Potassium			Growth
	Leaf fall	Spring	Mid-summer	Leaf fall	Spring	Mid-summer	Winter 1969-70
	(%)	(%)	(%)	(%)	(%)	(%)	(inch)
Untreated . . .	2.09 a	4.34 a	4.03 a	0.45 a	1.89 a	1.83 a	22.7 a
Soil nitrogen .	2.05 a	4.61 b	4.44 b	0.51 a	1.67 a	1.65 a	23.0 a
Foliar nitrogen urea	2.38 b	4.46 ab	4.25 ab	0.51 a	1.67 a	1.51 a	22.3 a
Foliar nitrogen KNO_3	2.46 b	4.46 ab	4.22 ab	0.80 b	1.89 a	1.80 a	21.2 a

[x]Means in each column followed by unlike letters differ significantly ($P < 0.05$).

occur either from external salt residues accumulating on the foliage which cause injury because of the very high osmotic concentration that develops when the residues redissolve (e.g. in dew), or the injury may occur from foliar absorption when the spray contains one or more ions to which the species is specifically sensitive. Stone fruit trees are sensitive to salinity on both counts (Benson et al. 1966). They have low tolerance to high osmotic concentrations and Na^+ and Cl^- have specific toxic effects.

It is considered that the severe injury observed in these studies was due to absorption rather than external residues. If injury was caused by external residues, then the injury should have taken the form of necrotic spots or patches spread at random over the leaf surface with injury in the leaf tip region especially. Instead, the injury took the form of severe marginal necrosis with accompanying leaf roll. Such symptoms are characteristic of salt toxicity in stone fruit trees following root absorption (Benson et al. 1966) and Ehlig and Bernstein (1959) reported similar symptoms following sprinkler irrigation of stone fruit trees with water high in sodium and calcium chlorides and sulphates. They found that injury in plum trees occurred with a leaf sodium concentration of 0.2 to 0.7 per cent and Lilleland (1951) reported injury in peach at 0.5 per cent. Thus the sodium levels found in the present study of 0.39 (sweet cherry) and 0.14 (peach) per cent are consistent

with the view that injury occurred following absorption. The author could find no description of calcium or magnesium toxicity symptoms in the literature though one might expect them to be similar to other salt toxicities. The relatively low calcium and magnesium levels attained in the leaves on the basis of published standards (Benson et al. 1966; Leece 1967) however are not consistent with the view that toxicity followed absorption, although some absorption clearly occurred.

On the other hand, consideration of spray osmotic concentration values for the various treatments tends to rule out external residues as the cause of injury. On this basis (Table 17) injury should have occurred as follows: $K^+ = Na^+ > Ca^{+2} = Mg^{+2} > NH_4^+ >$ Urea. However, the observed order in sweet cherry trees was $Na^+ > Ca^{+2} > Mg^{+2} > NH_4^+ =$ Urea $> K^+$. Thus K^+ and to some extent Mg^{+2} did not cause the degree of injury predicted on the basis of osmotic concentration. It might be argued that injury was caused by external residues except in the case of K^+ and Mg^{+2} which were partially absorbed. However this contention is not supported by the visual symptoms of injury.

If then it is concluded that carrier ion absorption occurred and that this in turn produced the observed leaf injury, the absence of injury with K^+ sprays must be attributed either to little K^+ absorption or to the less toxic nature (or greater metabolic need for) K^+. The latter

Table 17.--Approximate osmotic concentrations[x] of foliar sprays used in the cation evaluation experiment.

Compound	Molarity	Ionization Factor	Osmotic concentration
	(M)	(i)	(bar)
Urea	0.1	1	2.47
NH_4NO_3	0.1	2	4.95
KNO_3	0.2	2	9.89
$NaNO_3$	0.2	2	9.89
$Ca(NO_3)_2$	0.1	3	7.42
$Mg(NO_3)_2$	0.1	3	7.42

[x]Estimated using the van't Hoff equation (Salisbury and Ross 1969):

$$\pi = miRT$$

where: π = osmotic concentration (bars)
m = molality of solution
i = degree of ionization of compound
R = universal gas constant (0.083 litre bars/mole degree)
T = absolute temperature.

For the estimation, molarity was substituted for molality, it was assumed that the ionic compounds were completely ionised, and a temperature of 25° C (T = 298° A) was used.

seems the more likely explanation as the leaf analysis data indicated considerable K^+ absorption.

As none of the nitrate sprays raised leaf nitrogen concentration significantly and as K^+ was the carrier ion causing least injury, subsequent evaluations of nitrate foliar sprays were confined to KNO_3 and at higher concentrations than used in this experiment.

In light of the many reports of the effectiveness of urea sprays on crops other than stone fruit (e.g. Wittwer et al. 1967) it is noteworthy that the urea control was the only treatment to increase leaf nitrogen concentration in sweet cherry trees. It is also interesting that peach leaves were less susceptible to injury than sweet cherry leaves. This suggests cuticular differences between the species, the peach cuticle being more resistant to spray penetration than the sweet cherry cuticle, although it could also mean that the peach was less sensitive to salt toxicity than the sweet cherry. Both, in fact, may be true; however, the leaf analysis data, which showed increased leaf nitrogen and potassium concentrations produced by the urea and KNO_3 treatments respectively in sweet cherry trees in contrast to peach trees, support the penetration hypothesis.

Absorption and Translocation Experiment.--Having failed to demonstrate nitrate absorption in the carrier ion experiment this aspect of the experiment was repeated

using double the concentration of nitrate under more controlled conditions (greenhouse). A more sensitive indicator of absorption than leaf nitrogen concentration viz. induction of leaf nitrate reductase activity, was employed, and apricot trees were used as they were more sensitive to enzyme induction than other Prunus species (see the preceding section). This time nitrate absorption and initial metabolism occurred, however the increased spray concentration produced leaf injury the nature of which indicated salt toxicity following absorption. This injury was considered responsible for the stunting of subsequent terminal shoot growth and the failure to demonstrate translocation of nitrogen or potassium from the sprayed leaves to the new growth.

In summary, the sand culture experiments showed that K^+ was likely to be the most effective carrier ion for nitrate foliar sprays when applied to stone fruit trees and that absorption of both the carrier ion and the nitrate occurred. Depending on the carrier ion and the species injury occurred at a nitrate concentration of 0.2 to 0.4 g. equiv. per litre. Sodium ions and Ca^{+2} caused the most injury at equivalent nitrogen concentrations, Mg^{+2} was intermediate and K^+ caused least injury. Apricot seemed to be the most sensitive species to injury, sweet cherry was intermediate and peach was least sensitive. This

range of decreasing sensitivity may reflect increasing cuticular resistance to foliar penetration.

Field Experiments

A major question left unresolved by the sand culture experiments was whether sufficient nitrate could be absorbed from nitrate foliar sprays by the leaves to influence tree growth and fruit development. Field evaluations of nitrate foliar sprays were undertaken in an attempt to resolve this point. It was necessary to conduct these evaluations concurrently with the sand culture studies and to some extent this was unfortunate. In retrospect apricot or sweet cherry trees may have been more sensitive indicators of absorption and utilization than peach trees, and the initial nitrogen level chosen of 0.18 g. equiv. per litre seems too low. Nevertheless this rate was three times the rate that was being used commercially and in the second season the rate was increased to 0.4 g. equiv. per litre. The selection of K^+ as the carrier ion (on the basis of preliminary phytotoxicity studies) proved fortunate.

In both seasons autumn soil nitrogen applications corrected the nitrogen deficiency of the young commercial peach trees used in this experiment without, at the same time, breaking terminal bud dormancy in autumn and causing unwanted terminal growth susceptible to winter injury. In contrast autumn and/or spring nitrogen foliar sprays (either

as KNO_3 or as urea) at concentrations six times that used commercially did not affect the nitrogen status of the trees. Further the soil nitrogen treatment increased both tree terminal growth and trunk circumference the following season, which again were not affected by the sprays. It is thus concluded that insufficient nitrogen was absorbed from the foliar sprays to be able to influence tree nitrogen status or growth. Similarly with the carrier ion K^+, insufficient K^+ was apparently absorbed to influence tree potassium status or growth.

On the basis of these findings, it would seem doubtful that nitrogen foliar sprays would benefit other Prunus species. It would seem almost certain that the commercial practice of using nitrogen foliar sprays on stone fruit trees at a nitrogen concentration of 0.075 per cent (i.e. equivalent in nitrogen to a 0.6 per cent KNO_3 spray) would have no influence on tree nitrogen status or growth.

It also follows that further studies directed towards evolving a nitrate foliar spray suitable for stone fruit trees should center on examining surfactants and other spray additives that may improve leaf absorption of the spray. A sensitive assay system is provided by the induction of nitrate reductase in apricot leaves, either as used in the greenhouse in the sand culture studies reported herein or by modifying the greenhouse assay for

laboratory use. The nitrate reductase assay is rapid and avoids the criticism of nitrate or Kjeldahl nitrogen analyses that surface adsorbed nitrate, not removed by washing, may have been included in the analysis.

During the course of these studies, a modification for laboratory use of the greenhouse absorption assay was developed, based on the leaf disc technique of Sargent and Blackman (1962). It is presented as Appendix B. This leaf disc technique would permit a detailed study of the factors affecting spray penetration as well as the initial evaluation of spray additives which might enhance absorption. Preliminary studies undertaken using the leaf disc technique (Appendix B, Table 20) showed that partial removal of epicuticular waxes from field-grown apricot leaves enhanced cuticular penetration by 0.4 M KNO_3. This adds to the evidence presented in the Review of Literature that epicuticular waxes may reduce cuticular penetration by nutrient sprays in Prunus spp.

The finding in the sand culture studies that urea was the only compound to increase sweet cherry leaf nitrogen concentration, together with the finding in Field Experiment 2 that urea and KNO_3 produced similar increases in leaf nitrogen immediately following application in autumn, suggests that renewed studies of urea as a nitrogen foliar spray for stone fruit trees are warranted. The nitrogen concentration of urea is 3.5 times that of

KNO_3, and, now that a low biuret form of urea is available for spray purposes, urea could probably be applied to peach trees at more than double the concentration used in the second field experiment. In contrast, KNO_3 was being used close to its phytotoxic limit.

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APPENDICES

APPENDIX A

METHODS OF EXPRESSING CONCENTRATION

The various methods used to express the concentration of nutrient solutions in plant nutrition investigations have been reviewed by Hewitt (1966). In reports of orchard evaluations of foliar sprays, spray concentration is almost always expressed on a weight of chemical per volume of solvent (water unless otherwise stated) basis, a form which is very convenient from a commercial viewpoint. Three methods are in use. European workers express concentration on a per cent basis (i.e. g. per 100 ml.). Most British workers use pounds per 100 Imperial gallons and most American workers use pounds per 100 United States gallons. The relationship between the three units is as follows:

1 per cent ≡ 10 pounds per 100 Imperial gallons

≡ 8.345 pounds per 100 U.S. gallons.

As these methods, particularly the pounds per 100 gallons systems, lead to confusion when used interchangeably, reports of all field experiments have been expressed on a per cent basis in this thesis. In reviewing other work the author's units of concentration have been converted to per cent where necessary. Table 18 provides a quick

reference for conversion from pounds per 100 gallons (either Imperial or United States) to per cent, for the concentration range common in nutritional sprays.

Table 18.--Table for conversion from pounds per 100 Imperial or United States gallons to per cent, for the concentration range common in nutritional sprays.

Pounds per 100 gallons	Per cent	
	from Imperial gallons	from U.S. gallons
1.0	0.10	0.12
5.0	0.50	0.60
10.0	1.0	1.2
20.0	2.0	2.4
30.0	3.0	3.6
40.0	4.0	4.8
50.0	5.0	6.0

While the above weight per volume system of concentration expression is very convenient as far as the commercial preparation of sprays is concrened, it tends to be misleading when two or more sprays are being compared unless they happen to contain the same percentage of the active ingredient in question, in this case nitrogen. To facilitate comparisons on an active ingredient basis, concentration in the reports of the greenhouse and laboratory experiments has been expressed either on a molar or a gram-equivalent basis. The relationships among the various methods of expressing concentration for the compunds studied in this thesis are given in Table 19.

Table 19.--Relationships among the various methods of expressing concentration for the compounds studied in this thesis, when equated on a nitrogen basis to a 0.6 per cent urea spray.

Compound	g. equiv. N.	M	g. per litre	%	lb. per 100 Imp. gal.	lb. per 100 U.S. gal.
Urea	0.2	0.1	6.006	0.60	6.0	5.0
NH_4NO_3	0.2	0.1	8.005	0.80	8.0	6.7
KNO_3	0.2	0.2	20.222	2.02	20.2	16.8
$NaNO_3$	0.2	0.2	16.998	1.70	17.0	14.2
$Ca(NO_3)_2 \cdot 4H_2O$	0.2	0.1	23.615	2.36	23.6	19.7
$Mg(NO_3)_2 \cdot 6H_2O$	0.2	0.1	25.643	2.56	25.6	21.4

APPENDIX B

A TECHNIQUE FOR STUDYING NITRATE PENETRATION THROUGH INTACT LEAF CUTICLES IN PRUNUS SPP.

It became clear during the foregoing studies that stone fruit trees were able to metabolise nitrate in their leaves yet were unable to benefit from nitrate foliar sprays. It was concluded that insufficient nitrate was entering the leaves to be able to influence growth. A technique for studying nitrate pentration through intact leaf cuticles was developed, then used to test the hypothesis that epicuticular waxes were reducing cuticular penetration by nitrate ions.

Materials and Methods

Plant Material

Mature, mid-shoot leaves were obtained from 5-year-old apricot trees (Prunus armeniaca L. cv. "Curtis") growing at the Horticulture Research Center, Michigan State University, during the summer of 1970. The trees were in good vigor and had received 0.5 lb. nitrogen fertilizer per annum. The leaves were transferred to the laboratory in polyethylene bags at 0^{o} to 4^{o} C.

Methods

The technique developed for studying nitrate penetration through intact cuticles was based on induction of nitrate reductase in excised leaf discs following cuticular penetration. Using nitrate reductase induction as an index of penetration was preferred to using nitrate-nitrogen or Kjeldahl-nitrogen determinations because nitrate and Kjeldahl determinations would include not only that nitrate which had penetrated the cuticle but also that adsorbed on the surface. Washing techniques may not remove all the adsorbed nitrate.

Leaf discs from the apricot leaves were prepared for penetration studies using the technique of Sargent and Blackman (1962) as modified by Schönherr (1969). Glass cylinders (height 10 mm; external diameter 24 mm; internal diameter 21 mm) were attached to leaf discs (diameter 25 mm) using RTV 11 liquid silicone rubber (General Electric Company, Waterford, New York) hardened with Härter Tl catalyst (Wacher Chemie GMBH Company, Munich, Germany). The discs were placed in 9 cm. petri dishes lined with moist filter paper, 1.0 ml of the appropriate treating solution was added to each disc, then the dishes were incubated in a water bath at 25^{o} C, with a light intensity of 800 ft. candles (fluorescent) at leaf level. At the end of the incubation period the glass cylinders were peeled off the discs, the discs were rinsed with distilled water,

blotted dry, then assayed for nitrate reductase activity as described earlier.

Penetration was examined through the upper, astomatous surface only. The standard enzyme induction solution was 0.4 M KNO_3 and the control was 0.4 M KCl, each solution containing 0.1 per cent X77 surfactant (alkylarylpolyoxyethylene glycols, free fatty acids, and isopropanol--Chevron Chemical Company, Ortho Division, San Francisco, California). The standard induction time was 15 hours. Where required, partial removal of epicuticular waxes was achieved either by brushing the leaf with a camel's hair brush, or by wiping the leaf with a cloth dipped in 80 per cent (v/v) aqueous acetone, then drying the leaf immediately with an untreated cloth. Use of chloroform to remove the wax proved unsuitable in this system as it produced leaf disc senescence during the 15-hour incubation period.

Normally four discs could be obtained from each leaf without including major veins. This permitted four treatments to be compared at the one time. Each treatment comprised four discs (ca. 0.4 g. fresh weight tissue), one from each of four leaves, such that each leaf was represented by one disc in each treatment. Data were analysed by standard statistical procedures (Steel and Torrie 1960) and treatment means were compared by Tukey's ω-procedure.

Results and Discussion

Nitrate reductase was induced in discs excised from field-grown apricot leaves by 0.4 M KNO_3, using the technique described above (Table 20). When leaves were pretreated, either by brushing to re-orient the epicuticular wax, or by wiping with an acetone-soaked cloth to partially remove the epicuticular wax, enzyme activity induced by 0.4M KNO_3 was double that of non-pretreated leaves.

Table 20.--Induction of nitrate reductase in discs from field-grown apricot leaves, following cuticular penetration by 0.4 M potassium nitrate, as influenced by the partial removal of epicuticular wax.[x]

Induction System	Nitrate reductase activity ($m\mu$ moles NO_2^- formed,) (g. fresh weight^{-1}, hour^{-1})	
0.4 M KCl	217	a
0.4 M KNO_3	629	ab
0.4 M KNO_3--leaves brushed	1246	bc
0.4 M KNO_3--leaves acetone washed	1454	c

[x]Means followed by unlike letters differ significantly ($P < 0.05$).

It is concluded that the leaf disc technique would be a suitable system in which to evaluate spray additives in relation to cuticular penetration by nitrate ions. It

is also concluded that epicuticular waxes were reducing cuticular penetration by nitrate. It is possible that the action of acetone was not restricted to the epicuticular waxes but that some of the acetone penetrated the cuticle and reduced the resistance of embedded waxes to nitrate penetration. In the Review of Literature the available evidence indicated that the failure of peach trees to respond to urea sprays was caused by cuticular waxes reducing urea absorption. The data just presented indicate that cuticular waxes may also cause the lack of response exhibited by Prunus spp. to nitrate sprays.