THESIS

NITRIFICATION SUPPRESSION AND PROVITAMIN A
CONTENT OF SPINACH

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED

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ABSTRACT OF THESIS

NITRIFICATION SUPPRESSION AND PROVITAMIN A
CONTENT OF SPINACH

'Bloomsdale Longstanding' spinach was grown in the field and in growth chambers to determine the effect of soil NH_4 and soil NO_3 on spinach yield, green color, leaf NO_3, and provitamin A (carotene) content. Nitrapyrin (Np) was adsorbed onto $(NH_4)_2SO_4$ crystals and applied as a band in the field and blended with a soil-perlite mix in the growth chambers. Field treatments received $(NH_4)_2SO_4$ fertilizer at 90 kg N/ha and differed only by the addition of 140 g/ha (0.0625 ppm soil basis or 0.156% based on N) Np to one treatment. Significant increases in yield, green color, and carotene content were noted for the Np treatment in the field and attributed to increased soil N retention. A green color difference between treatments with and without Np in the field, as measured by a reflectance meter, of 0.05 relative units correspond with a 15% increase in carotene content for the Np treatment. Carotene yield (kg carotene/ha) of the Np treatment was 43% greater than from the same treatment without Np.

Growth chamber treatments varied soil NH_4-N and NO_3-N concentration while maintaining equal fertilizer N applications to expose spinach to a range of NH_4/NO_3 ratios in a nonleaching system.

Fertilizers for the four growth chamber treatments supplied equal amounts of N (400 mg N/2 kg soil mix) for each and were $(NH_4)_2SO_4$, protected with Np and unprotected, $Ca(NO_3)_2$, and a mix of protected $(NH_4)_2SO_4$ and $Ca(NO_3)_2$ to provide a 0.33 NH_4-N/NO_3-N ratio. Np rate was 3 mg/2 kg soil mix or 0.75% based on N. Ammonium toxicity symptoms and greenest color were noted for spinach grown with essentially all NH_4-N. Spinach receiving $(NH_4)_2SO_4$ as sole N source with or without Np, produced statistically equal as well as the greatest amount of carotene. A 48% increase in carotene yield of spinach resulted when NH_4 was the available source of N compared to NO_3. Large differences in leaf carotene correspond to small differences in leaf color in spinach.

Proper manipulation of the soil NH_4/NO_3 ratio by means of nitrification suppression can increase spinach yield and provitamin A content as well as improve green color with lower leaf nitrate thus bettering consumer acceptability.

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INTRODUCTION

The widespread use, increasing costs, and limited supply of nitrogen fertilizers provide impetus to the search for means to increase nitrogen fertilizer efficiency while maintaining crop yield and nutritional quality. Nitrogen transformations in the soil, especially the rapid conversion of NH_4 to NO_3 through nitrification make it difficult to maintain significant amounts of soil N in a relatively nonleachable form. Soil additives have been developed to control nitrifying activity of indigenous soil bacteria to provide a regulated supply of NH_4 and NO_3 in the soil. A chemical developed by the Dow Chemical Co. called N-Serve® with active ingredient nitrapyrin (Np) 2-chloro-6 (trichloromethyl) pyridine has been shown to selectively inhibit nitrifying activity of <u>Nitrosomonas sp.</u> bacteria. Used in conjunction with ammoniacal fertilizers, it is a valuable nitrogen management tool with application in research and commercial crop production.

Leafy vegetable crops take up large amounts of N in NO_3 form over very short periods of time. Spinach, for example, may take up as much as 134 kg N/ha from the soil in 21 days just prior to harvest (163). This necessitates maintenance of high soil N levels to prevent nitrogen deficiency. Loss of soil N by leaching is a problem especially when NO_3 fertilizers are used, though the potential for leaching

is also provided by NH_4 fertilizers due to nitrification under normal crop conditions. Previous studies indicate that Np, applied as an additive to $(NH_4)_2SO_4$ fertilizer can reduce leaching losses of N and produce yields comparable to NO_3 treatments in spinach. The ratio of NH_4 to NO_3 in the soil may be responsible for the influence on yield since different species have been shown to respond preferentially to NO_3 or NH_4. This study employs Np as a nitrification suppressant to maintain applied NH_4 fertilizers as NH_4 in the soil to investigate the effect of NH_4/NO_3 ratio on spinach yield, green color, leaf NO_3, and provitamin A content.

Human health hazards associated with maintenance of high NO_3 levels in the soil are ground water pollution through leaching and accumulation of NO_3 in plant tissue. Ingested NO_3 from plant tissue may be reduced to NO_2 and cause methemoglobinemia in infants. Concern is also focused on possible formation of carcinogenic nitrosamines from nitrites in the gastrointestinal tract in both infants and adults. Previous studies have shown NH_4 fertilizers to limit NO_3 accumulation in spinach tissue especially when used in conjunction with a nitrification suppressant. This study further investigates the effect of suppressed nitrification conditions in the soil on spinach NO_3 content.

Dark green leaf color is an important market quality of spinach. Nitrogen and other nutrient deficiencies must be avoided in crop production due to their chlorosis inducing effects. Growing spinach under

suppressed nitrification conditions, using Np and $(NH_4)_2SO_4$ fertilizer, has produced a darker green leaf color compared to using $(NH_4)_2SO_4$ without Np or NO_3-N fertilized treatments in the field. The exact mechanism by which nitrification suppression and the resulting preponderance of NH_4 darkens green color is not known. The current study further investigates the effect of suppressed nitrification on spinach leaf color.

Green color of leaf tissue is considered by some researchers to be associated with provitamin A (carotene) value though the exact relationship is not known. Vitamin A does not occur in plants. Provitamin A is metabolized by animals to form vitamin A. Carotene is one of the many carotenoids found in green leaves. Spinach tissue carotene is approximately 90% beta-carotene. Beta- and alpha-carotene show the highest vitamin A activity in animals. In the United States, about 50% of the vitamin A intake in humans is in the form of provitamin A from plants (14). In India, attempts are being made to increase consumption of leafy vegetables as one method of improving vitamin A intake. Thus the carotenoid vitamin A precursors are an important source of vitamin A in the human diet. The provitamin A content has not previously been quantitatively associated with green color of plant tissue. Previous studies with herbicides and soil fumigants suggest that nitrification suppression may have a beneficial effect on carotene content of leaf tissue. This effect has not been

investigated in spinach.   The present study examines the influence

of suppressed nitrification on provitamin A content of spinach.

LITERATURE REVIEW

Nitrification Suppression and Soil

The control of soil pathogens such as fungi and nematodes has
brought about the development of many non-selective soil fumigants
which are toxic to soil nitrifying bacteria. Two separate reviews
noted that nitrifying organisms were more sensitive to soil fumigants
than ammonifiers (89,150). EDB (ethylene dibromide) (94,139),
Picloram (56), Cloropicrin (134), methyl bromide (154,160), and
Nemagon (160) all have inhibitory effects on nitrifying bacteria in the
soil. The broad spectrum soil fumigant D-D (133) and the nemacidal
fumigant Telone (154), applied at recommended rates, inhibited
nitrification for 8 weeks. EDB, at recommended rates, reduced soil
nitrifiers enough to reduce at-planting ammonium fertilizer recom-
mendation by one-half (139). In fact, NH_4 toxicity problems have been
attributed to low nitrifying bacteria populations when Telone was used
at recommended rates on celery (154).

Attempts have been made to develop soil additives which are
specifically toxic to nitrifying Nitrosomonas sp. bacteria. A recent
review covering the effects of thiourea, methionine, dicyandiamide
(cyanoguanidine), and a number of urethanes concluded that none of
the chemicals tested were as effective or as specific as nitrapyrin(54).

Nitrapyrin has been shown to be nontoxic to <u>Nitrobacter sp.</u> (54), other species of bacteria and fungi (55), algae (56), and many plant seedlings (120). The phytotoxicity of Np has been estimated as being between 50 and 100 ppm (54,97), though direct toxic effects of Np are unclear. Nitrapyrin is most often used with anhydrous NH_3 (70,100, 106) though its inhibiting effect on nitrification has been noted using NH_4NO_3 (140), aqua NH_3 (141), urea (55), and $(NH_4)_2SO_4$ (98,100,113).

Due to the specificity of Np for <u>Nitrosomonas sp.</u> and the sensitivity of the bacteria to soil fumigants, very small concentrations of active Np have noticeable effects on the nitrification process. The result of varying Np concentration when applied to $(NH_4)_2SO_4$ fertilizer is to hold applied NH_4 in NH_4 form for varying lengths of time. The recommended rate of application for active Np is 1-2% of applied N rate. Varying degrees of nitrification inhibition have been noted at active Np application rates of 0.5% to 2.0% of the fertilizer N (141), with higher rates causing more severe inhibition (54,67). The effect of Np is also dependent on soil texture (141) and soil organic matter content (54).

The slow release effect of using Np with an NH_4-N fertilizer makes possible the elimination of side-dress applications of N during the growing season for leafy vegetables (100), including spinach (140), without sacrificing yield. Fall fertilizer application efficiency may also be increased by limiting NO_3 formation and subsequent denitrification or leaching between growing seasons (49,66,67).

A possible disadvantage of using Np with NH_4 fertilizers is ammonium toxicity in susceptible species. High concentrations of NH_4 in the growing medium may cause curling and burning of leaf margins in beans (13), lesions on stem and leaf tissue in tomato (8,146), and dark coloration and decreased size of roots (15,76,91). Tolerance to water stress is also lowered due possibly to membrane impairment or structural damage, a decrease in osmotic potential, or prolonged stomatal closure (110). Spinach ammonium toxicity symptoms have been reported as decreased growth measured by fresh and dry weight (96), and a dark green leaf color with severe stunting and necrosis (113). The exact mechanism by which NH_4 nutrition may cause ammonium toxicity symptom expression is not understood though several theories have been developed. Nitrapyrin alone did not affect green color or induce ammonium toxicity symptoms in spinach grown in the field (113). Nitrapyrin, applied at recommended rates, had no detrimental effect on growth chamber spinach receiving all NH_4 fertilization indicating no direct toxic effect of Np (98).

The pH of growing media is decreased under NH_4 nutrition through nitrification (76), which may have detrimental effects on growth by disrupting rhizosphere ionic balance. If neutral pH of growing media is maintained, equal growth of beans (9) and other species (8,121,149) is noted regardless of N source. The pathways of NH_4 assimilation seem to be pH sensitive and more effective at neutral to alkaline soil pH.

DeWit (39) indicated NH_4 toxicity may be due to stress on normal cellular organic acid content since more intercellular organic acids are required with NH_4 nutrition to balance the increase in cellular pH. This argument is debatable since cellular organic acid levels have inconsistent effects on plant growth and yield (138). Ammonium may also inhibit ATP production and photosythetic electron transport systems (117). Plants with large amounts of carbohydrate reserves such as sweet potato and onion seem to tolerate high soil NH_4 levels. This may be related to the capacity of the plant to detoxify NH_4 by amination (42,154).

Nitrification results in acidification of the soil solution. Studies comparing NH_4 and NO_3 nutrition have shown lowered media pH when NH_4 provides the N source and nitrification is nonexistent (10,74,76, 91). Ammonium nitrogen enters the plant by absorption or through active uptake carrier mechanisms in root cell membranes. When NH_4 is absorbed by the plant, H ions are given off by the root to maintain electrochemical neutrality resulting in an initial increase in cellular pH (23,40). However, assimilation of NH_4 into organic forms within the plant yields two acidifying reactions. Ammonium combines with glutamic acid to form glutamine and with CO_2 and ATP to form carbamyl phosphate (117). Hydrogen ions are released during both reactions which are either recombined within the cell or released into the media solution. Activated membrane ATPase, dissociating

water and establishing a pH gradient with H ions accumulating outside the cell, and the exchange of internal H ions for NH_4 from cation carrier mechanisms may be causes of media pH decreases (65). Therefore, with the naturally acidifying reaction of nitrification suppressed or nonexistent as in solution cultures, a decrease in media pH may still be expected.

A decrease in medium pH as a result of metabolic or uptake processes is most pronounced in the rhizosphere (122). This pH decrease may be responsible for increased uptake of Fe (102,113) and Zn (78) since these micronutrients exist mainly in oxidized forms when media pH is neutral or high (21). A decrease in K uptake under NH_4 nutrition has been noted (37) and attributed to competition for absorption sites (156). This is not a problem in western alkaline soils where available soil K is usually abundant. Lower rhizosphere pH resulting from NH_4 nutrition has also been shown to increase absorption of P, perhaps by facilitating soil phosphorous dissolution (95). Hence, availability of certain nutrients is altered under ammonium nutrition regimes and may influence spinach yield, green color, NO_3, and provitamin A content.

Yield

Though NH_4 toxicity problems may develop, the use of Np with NH_4 fertilizers is a promising tool to maximize fertilizer efficiency and in so doing increase yields in spinach. Spinach seems to utilize

NH_4 during early stages of growth but produces highest yields when NO_3 is available during its exponential phase of growth (113). This phenomenon is characteristic of other leafy vegetables (127). It has been demonstrated that maximum spinach yields are attainable by eliminating sidedress applications of N fertilizer when Np is used with at-planting NH_4 fertilizers (100, 140). In the latter study, increased soil retention of N, i.e. leaching prevention, due to slowed nitrification of NH_4 fertilizers was cited as being responsible for the results. In another field experiment, treatments receiving $(NH_4)_2SO_4$ at 140 kg N/ha with 0.0625 ppm Np produced yields comparable to treatments receiving 280 kg N/ha as $(NH_4)_2SO_4$ without Np (113). Hence, the use of Np with ammonium nitrogen fertilizers may be suitable for increasing fertilizer efficiency in spinach production.

Manipulating the ratio of NH_4 to NO_3 with regard to fertilizer application positively affected yields in certain crops. Adjusting the NH_4/NO_3 ratio to provide at least some of the N as NO_3 was shown to be superior to all NH_4 fertilizer sources for kale (127), corn (119), pea (7), and cucumber (158). When Np was used to maintain applied NH_4 fertilizers in NH_4 form, a NH_4/NO_3 ratio of 0.33 was shown to be as effective as NO_3 treatments in producing maximum spinach yields in the growth chamber (98) and the greenhouse (114). When NH_4 supplied up to one fourth of the total N applied as nutrient solution to Np (50 ppm) treated soils for spinach grown in the growth

chamber, maximum yield was obtained and was not significantly different from NO_3 fertilized treatments (98). Hence, adjusting the NH_4/NO_3 ratio using a nitrification inhibitor, may be an effective means to increase N fertilizer efficiency.

Spinach Tissue Nitrate Content

The nitrate content of spinach merits concern due to its possible reduction to NO_2 and consequent effects on human blood cells (methema-globinemia in infants) (33, 148), muscle tissue (79), and the formation of carcinogenic compounds (144). A recent review of literature points out that it is subsequent reduction of NO_3 to NO_2 after ingestion which leads to possible toxic effects (96) and therefore NO_3 content of edible tissue is of concern. Excessive levels of plant NO_3 have been attributed to liberal application of N fertilizers to maintain adequate N in the root zone for fast growing crops (12, 33, 92, 145). Ground water pollution also results from excessive N applications in the field (145).

The accumulation of NO_3 in spinach varies among cultivars (11) and is enhanced by high temperatures (33) and low light intensity (35). However, the overriding factors influencing NO_3 accumulation in spinach are the timing, amount, and source of N fertilization (96). A direct relationship has been observed between the quantity of NO_3 supplied in the fertilizer and spinach NO_3 concentration (35, 92). Simultaneous injection of a nitrification inhibitor and anhydrous NH_3

into the soil has been shown to decrease NO_3 accumulation in head lettuce grown in the field with no sacrifice in yield (100). Nitrate in spinach tissue was lowest when NH_4 supplied all of the N, especially when Np was used. Tissue NO_3 increased with increases in fertilizer concentration of NO_3 (98). Hence, Np may offer a means to avoid high levels of NO_3 in spinach tissue without sacrificing quality or yield.

Spinach Green Color

Ammonium nutrition has a tendency to induce darker green leaf color compared to NO_3 nutrition, though the exact mechanism by which this occurs is not understood. An increase in green color implies an increase in chlorophyll content. Nitrogen availability plays an important role in maintaining green leaf color (58, 90) as do micronutrient availability (45) and soil pH (64). Studies comparing NH_4 nutrition with NO_3 nutrition have shown darker leaf color with NH_4 nutrition with tomato (87), pineapple (133), and corn (62, 78). When NH_4 nutrition was combined with Np to maintain soil N as NH_4, darker green color of leaf tissue from corn (70), lettuce (100), and spinach (113) was noticed compared to no Np treatments.

The increased chlorophyll content, and hence darker green color of NH_4 fed plants may be due to increased Fe utilization within the plant. In a solution culture experiment, the chlorophyll mutant "xantha$_5$" of _Lycopersicum esculentum_ was used to study the effect of

NH_4 and NO_3-N sources on leaf chlorophyll content (87). Solutions

with NH_4 raised the chlorophyll content 100-200% in comparison to

NO_3 treatments. Several hypotheses concerning the beneficial effect

of NH_4 nutrition were discounted by the author. The increase in

chlorophyll formation as a result of NH_4 treatments was not due to an

increase in Fe uptake since plants containing the same iron content

often differed significantly in their chlorophyll content. The pH of

leaf tissue was also discounted since buffered NH_4 treatments raised

leaf tissue pH but did not increase the influence of NH_4 on chlorophyll

formation. In the same experiment, ^{15}N was used to show that the

uptake and metabolism of NO_3 and NH_4-N sources and their input into

protein was not blocked in the mutant. Hence, the differential influence

of NH_4 on chlorophyll content was not due to a differential ability of

the mutant to utilize NH_4 or NO_3.

The conclusion and resulting hypothesis reached in the study was

that NH_4 and NO_3 changed the redox potential of the cell in different

ways which had a direct influence on the form of Fe within the plant.

Although uptake of NH_4 induces an initial increase in cellular pH,

assimilation into organic compounds eventually decreases cellular pH

thus influencing Fe to be in an unoxidized form whereas cellular NO_3

may act as an oxidizing agent. Iron utilization may be the factor

limiting chlorophyll formation in NO_3 fed plants, and is overcome when

plants are supplied with NH_4.

Spinach Carotene Content

Green color of leaf tissue has often been associated with its value as a food source, especially with respect to provitamin A (carotene) content. Vitamin A is not found in plants. Animals convert provitamin A from plant tissue to vitamin A (retinol) through metabolism. Unsubstantiated claims pertaining to the relationship between greenness of leaves and content of carotene are numerous and suggest that the greener the leaf, the higher its carotene content (1, 14, 44, 82, 141, 142, 151). The ratio between chlorophyll content and carotenoid content varies widely among green plant species (111) making establishment of quantitative relationships difficult. Carotenes, like the chlorophylls, are confined to the chloroplast of the green cell though different pathways of biosynthesis, under control of different regulatory genes have been reported (73). Therefore, the degree to which carotene content parallels that of chlorophyll is not necessarily constant and remains to be determined.

Some investigations have shown that carotene content of leaves parallels chlorophyll content to some extent. Bernstein (16) noted, as did Hamner (60), that conditions inducing chlorosis in leaves will decrease their carotene content. A similar relationship was found to exist for green leaves when compared to senescent autumn leaves (129). Using a qualitative color measurement, the greenness of greenhouse grown spinach was associated with carotene content when

nutrient deficiencies were induced (31). When etiolated barley seed-
lings were exposed to red light, increases in both chlorophyll and
carotene were noted (130) though a quantitative relationship between
green tissue color and carotene content has not as yet been established.

Several reviews have been made of the influence of the environ-
ment on the carotene content of plants (14,28,46,61,93,125). Aspects
considered as influential include plant maturity, growing temperature,
light, storage conditions, herbicides, soil moisture, and fertility.
This review will consider soil fumigation as part of soil fertility due
to the effect of certain fumigants on soil nitrifying bacteria. Diffi-
culty in establishment of constant relationships between these factors
and plant carotene content arises from natural variation of carotene
concentration within the plant (24,137) and is further compounded by
the influence of uncontrolled environmental factors as discussed
below.

The carotene content of leaves is influenced by the maturity of
the leaf tissue, though the exact mechanisms involved are not known.
As the plant matures, the beta-carotene molecule, of greatest activity
relative to provitamin A, changes in form within the plant (36). The
beta-carotene molecule is the most unstable carotenoid and its form
and association within the plant influences its stability with regards to
isomerization and oxidation (19,49). Generally, the carotene content
of green leaves increases with maturity and peaks at flowering and

then decreases (46). In alfalfa, leaf carotene increases with maturity

until just before flowering and then decreases in concentration (86).

Variation of the time between cuttings of alfalfa illustrated this point

by showing that the shorter the time period between cuttings, and

hence the less mature the tissue, the higher was the carotene content

(34). The carotene content of spinach leaves has been found to de-

crease with maturity (131). The increase of carotene with maturity

is probably true of spinach leaves though chlorophyll may have a

masking effect by influencing isomerization (36). Analysis of turnip

greens demonstrated that younger leaves had a higher concentration

of carotene than did older leaves on the same plant (124). Maturity

must therefore be considered in experiments investigating influences

on carotene content of green leaves.

Carotene content of green leaves responds to light intensity of

the growing environment (105). Etiolated leaves grown in the dark

have less carotene than leaves grown in the light (53). The same

pattern holds true for inner, light green leaves of head lettuce com-

pared to outer, green leaves (1). Comparing full sun with shaded

growing conditions, one finds more carotene in shaded corn (101) and

turnip greens (126). Bodea (26) analyzed plants from altitudes of 0

to 2000 meters and noted lower beta-carotene concentrations at higher

elevations. Comparing the field results with studies in vitro, he

attributed the results to increased ultra-violet light exposure at high

altitudes inducing beta-carotene epoxide formation. This conclusion is supported by other investigators (52). Beta-carotene epoxides have very low vitamin A activity in animals (69) and are usually found in extremely low concentrations if at all in green leaves (50). The ratios of different carotenoids also varies with photosynthetic conditions (102).

Temperature of the environment also influences carotene content of leaves. Investigations have shown that leaf carotene concentration increases with temperature in corn (101) and in detached bean leaves (5). A Q_{10} for carotene synthesis over the $25^{\circ}-35^{\circ}C$ temperature range was established as 1.4 in detached bean leaves. Palmer (105) reviewed German literature and cited temperature and light as controlling factors on carotene synthesis explaining that low temperatures and diffuse light environments produced most carotene in leaves. Temperature and light conditions may influence the results in plant provitamin A investigations.

Storage conditions including temperature, relative humidity, and atmosphere influence carotene content. When storage temperature was decreased to $0^{\circ}C$ from $10^{\circ}C$, a three fold increase in retention of carotene in green tissue was observed (28). A decrease in storage chamber relative humidity to 65% from 95% caused a 64% greater destruction of carotene in head lettuce (63). Since beta-carotene is very susceptible to oxidation in fresh leaf tissue (36), the oxygen

content of storage atmospheres can play an important role in affecting carotene content (51,72). When oxygen was bubbled through suspensions of chloroplasts, a 27% increase in beta-carotene destruction was noted (48). High nitrate content of leaf tissue, and more importantly, its possible subsequent formation of nitrite may cause severe beta-carotene losses in storage (103).

Drying of leaf tissue, especially in forage crops such as alfalfa, may cause severe losses of provitamin A (85). Heating of leaf tissue prior to dehydration may be an effective means to prevent carotene losses since carotenes dissolve in intracellular oil-droplets during heating and in this way are protected from oxidative breakdown during dehydration (41). It has been demonstrated that total carotene concentration of alfalfa does not decrease as drying temperatures increase, but an increase in carotene stereoisomer formation does occur, thus lowering the provitamin A activity of the forage (85). Specific factors influencing carotene steroisomer formation within the plant are not as yet understood. Precaution should be taken to insure uniform treatment of plant samples in carotene content comparison studies.

Investigations have been undertaken to ascertain the effects of herbicides on plant carotene content though consistent relationships have not been established. In an extensive study of sweet potato foliage and roots, Grieg (57) found applications of Amiben, Randox (DCAA), Dacthal (DCPA), Dymid, or Treflan to have no adverse effect on

foliage carotene content. A similar null effect on carotene content
was observed when CDEC or endothal was applied to spinach (131).
The latter investigator noted that the chlorophyll molecule might
mask the effect of herbicide on spinach. Applications of 2,4,-D to
1 month old buckwheat plants caused a decrease in carotene content
of stems and leaves compared to unsprayed plots (157). Coastal
Bermudagrass carotene content increased when Simazine was used to
control broad leaf weeds but results were not consistent at differing
levels of N fertilization (99). Simazine was found to have no effect
on nitrification (47). A consistent effect of herbicides on carotene
content of plants remains to be established.

Soil fertility has long been of concern with regard to influencing
provitamin A content of plants. Several review articles exist covering
influences of macronutrients, micronutrients, and soil physical and
chemical properties on carotene content of green leaves (52,93,125).
Generally, soil conditions inducing chlorosis will decrease carotene
content of leaves (16,31,46). Quantitative relationships are difficult
to establish due to factors previously mentioned. Soil fertility charac-
teristics including physical and chemical aspects, as well as form of
nitrogen in the soil, especially under suppressed nitrification condi-
tions, have potential for influencing provitamin A content of green
leaves.

The moisture content of soils has been shown to influence
carotene content of turnip greens (68) though this may not be true

when treatments are compared on a carotene per dry weight basis (112). Uniform soil moisture has been cited as necessary for uniform carotene content of coastal Bermudagrass which is important in the feed industry (152). In extensive studies on wheat (161) and oat (162) leaves, it was demonstrated that cation exchange capacity of soil and soil organic matter had primary influence on carotene content of the plants. Other aspects covered in the latter study, but shown not to influence carotene content, included percent base saturation, calcium carbonate, and exchangeable calcium and magnesium.

Micronutrient deficiencies have been shown to limit carotene production in green leaves. Supplying boron on deficient soil caused increases in carotene content of beet greens (59), alfalfa, and pasture grasses (107). Copper and manganese may also limit spinach and barley carotene production (46).

Macronutrient deficiencies have also been shown to limit leaf carotene content. A review of literature prior to 1953 (46) cited decreasing Ca as limiting carotene content of sand cultured oats, while calcium and magnesium had no effect on other species. The null effect of Ca (161) and Mg (34, 161) is supported elsewhere but is contradicted by a solution culture experiment with turnip greens which concluded that Ca deficient plants contained less carotene (16). In the same study, sulfur deficiency was also cited as decreasing carotene content of turnip greens.

The difficulty of establishing constant relationships where soil factors and nutritional quality are concerned is again illustrated by leaf carotene being shown to be affected in different ways or not at all by P concentrations in the growing medium. A solution culture study demonstrated that P deficiency actually increased carotene content of spinach but repeating the experiment in soil produced results indicative of deficiency having no effect (31). Eliminating P and K fertilizer applications to coastal Bermudagrass did not change carotene content with respect to P and K fertilized controls (34). In an exhaustive statistical study, solution cultured turnip green with S and K deficiencies showed less leaf carotene than control pots while P deficient treatments showed leaf carotene levels that were not different from nondeficient controls (16). Data obtained from a field study of rye grass produced a positive correlation between available P in the soil and carotene content of the leaves which the investigator attributed to the association of available P fractions with organic material in the soil (161).

The macronutrient most often cited as influencing leaf carotene content is nitrogen. Fernandez (46) cited seven studies in which increases in leaf carotene content were established. This pattern has also been noted in more recent work for coastal Bermudagrass (34, 99). In fact, doubling the fertilization rate for this species has produced as much as 16% greater carotene in leaf tissue (34). The form of soil

N has also been cited as being a possible influence on leaf carotene levels. Fernandez (46) concluded in his review that applied nitrate may be more effective in increasing leaf carotene levels while another investigator cited ammonium as being effective in increasing leaf carotene content of rye grass (123). When additional N in the form of nitrite (NO_2) was supplied to tobacco plants, the ratio of xanthophylls to carotenes in the leaves decreased and was attributed to a preferential synthesis of carotene (118). Applications of N in organic form as horse manure produced leaf carotene levels in rye grass that were not different from inorganically fertilized plots (29).

The influence of nitrification suppression on green leaf color and the nebulous relationship between green color and provitamin A content have been discussed. Although an increase in green color may imply an increase in carotene content, to this date no studies have investigated nitrification suppression per se and its effect on leaf carotene content. Studies do exist which indicate that suppressed nitrification may have a positive influence on carotene synthesis though the exact mechanisms involved are not known. When soil fumigants Telone and Nemagon were used prior to growing carrots, an increase in carotene content of the roots was noted (43, 118, 160). An increase in carotene up to 16% above unfumigated control plots has been achieved (118). Several theories have been postulated (159). One investigator (118) cited suppressed nitrification, caused by the fumigants (89, 134, 139, 150, 154), as increasing carrot carotene content due to increase in

NH_4 availability in the soil. The question of whether this phenomenon may affect leafy vegetables such as spinach in a similar manner yet remains to be answered.

Carotene Analysis

The majority of research dealing with carotene content of dried plant materials has been associated with the nutritional quality of dried plant meals used for chicken feed including alfalfa, grass, corn gluten, and marigold petals. Carotenoids, especially certain xantho-phylls (carotenoids containing oxygen), play a major role in avian skin and egg yolk pigmentation (105). Carotenoid concentration in dried feeds is of particular concern when evaluating feed drying techniques (85) and uniformity of feed products (34).

Xanthophylls and carotenes (carotenoids containing no oxygen) comprise the bulk of extractable yellow pigments of green leaves (51). Beta-carotene is utilized most efficiently by animals in vitamin A synthesis (69) and is the most abundant carotene in green leaves (14). Xanthophylls are found in approximately double the concentration of carotenes in fresh plant tissue (82) and exhibit light absorption charac-teristics very similar to carotenes (51,69). Due to the relatively low provitamin A activity of xanthophylls (14), it is desirable to separate carotene and xanthophyll fractions of extracts when investigating pro-vitamin A value of spinach.

It is recognized that obtaining consistent results of carotenoid analysis of dry plant tissue is difficult (136). Carotenes are susceptible to oxidation in air (85), light (51), and heat (28). The amount of carotene remaining in dried tissue samples varies widely and depends on temperature and duration of drying and duration of air and light exposure (24,51) especially ultra-violet light (26). It has been estimated that as much as 33% of the carotene in fresh alfalfa tissue may be lost during drying (85).

To compare nutritional quality of spinach grown under different nitrogen regimes, an accurate, quantitative method of carotene analysis is necessary. Differences in carotene content, if consistent with green color, were expected to be small if detectable. A method yielding the maximum amount of carotene with a low coefficient of variability was sought.

Thin layer chromatography has been used primarily in qualitative separation of carotenes from plant tissue (38,80) though a quantitative method has been developed for dried alfalfa (71). Carotenoids are extracted from 2 grams dried tissue in stagnant hexane-acetone-methanol (30+10+5) for 16 hours. The extract is concentrated 5:1 under reduced pressure and 100 to 200 ml are streaked on silica gel coated plates and placed in vapor tight tanks containing solvents to accomplish separation. Carotenes, being less polar then xanthophylls, show a higher R_f value in benzene-methanol (85+15) developing solvent

and form distinct bands. Carotene bands are scraped off and eluted in acetone. The solution is mixed and centrifuged and absorption is immediately determined at 436 nm. Comparison is made to standard solutions of 90% beta- and alpha-carotene. Although extraction and separation of xanthophylls and carotenes from alfalfa showed consistent results, attempts with dried spinach in this study yielded a high coefficient of variability of 16.1%. This method was abandoned in favor of a modification of the method currently approved by the Association of Official Analytical Chemists (AOAC) for carotene analysis of dried plant materials employing column chromatography (84).

The efficacy of column chromatography in separation, purification, and isolation of carotenes, since its development by Tswett in 1906 (69), has been well recognized and has been effectively used for quantitative analysis of carotenes in dried plant tissues (38). The current analysis procedure of AOAC and those procedures to be discussed all employ essentially identical chromatography materials and techniques to isolate carotenes after extraction. Focus of this review is therefore placed on extraction procedures to illustrate differences in extractable carotenes.

Current AOAC extraction procedure development has followed the goals of maximum extraction of pure pigmenting carotenoids while maintaining low variability. Speed of analysis is also a concern in

forage quality control determinations (84). The groundwork of the present AOAC extraction procedure has been laid by Kohler (77) in an attempt to provide a simple chromatographic method which could be used in quality control laboratories. Previous methods had employed heating (56°C) during extraction though loss of carotene is noticed due to oxidation and isomerization (17,137). Kohler employed an ambient temperature, 16 hour, stagnant hexane-acetone (7+3) extraction of dried alfalfa hay ground to 40 mesh. Two ml methanolic KOH (40%) were added afterwards in addition to 2 ml water. The extractant is mixed briefly and allowed to settle 1 hour before chromatography. Reproducible results were obtained for stem, leaf, and whole alfalfa meals and for coastal Bermudagrass meal.

Quackenbush (109) developed the current AOAC (4) procedures by using cold saponification as proposed by Kohler and comparing hexane-acetone HA (7+3) extractant with hexane-acetone-ethanol-toluene, HAET (10+7+6+7). Though values for xanthophylls were equal or higher using HAET, carotene values for dehydrated alfalfa were higher using HA. Since xanthophylls are more potent avian pigmenters, the HAET extractant was adopted by AOAC (3) and to this date has remained unchanged (4).

Discrepancies were noticed by Livingston (83) between methods of Kohler (77) and AOAC (3) and attributed to differences in water levels during extraction. To facilitate extraction of the more polar

xanthophylls, Livingston modified Kohler's method by adding 0.5 ml water during the 16 hour stagnant extraction. Besides demonstrating increased extractable xanthophylls, higher carotene values than AOAC's (3) or Kohler's (77) method were noticed for sun dried alfalfa meal and dehydrated Bermudagrass.

The procedure for carotene analysis adopted for this study is a modification (84) of the AOAC (3) method. Developed for the feed industry, the method employs ambient temperature, agitated extraction and saponification which permits rapid analyses of dried spinach tissue. Addition of 0.5 ml water during extraction is included since prior investigation has shown it to be essential for complete extraction of most dehydrated plant meals (109). A one hour, stirred HA (7+3) extractant with 1 g dried, ground (40 mesh) tissue replaces the 16 hour soak as in the AOAC (3) method. Total carotenes are isolated using column chromatography (3) and absorbance is determined at 436 nm. Calculations of mg total carotene per kg fresh spinach tissue are based on absorbance readings from standard solutions of 90% beta- and 10% alpha-carotene.

METHODS AND MATERIALS

Field Experiment 1975

A previous field experiment by Riggert (113) provided spinach

tissue, from two treatments, to analyze for carotene content. The

experiment was located at the San Luis Valley Research Center near

Center, Colorado. The field had been planted to wheat the previous

year. Table 1 lists the chemical and physical characteristics of the

soil. Prior to planting and treatment application, a leaching pro-

cedure was used to lower residual soil NO_3 levels. A broadcast appli-

cation of 250 kg P/ha was incorporated into the soil to supplement

residual soil P.

Two treatments were selected for carotene analysis on the basis

of maximum differences in leaf color measurements and were identical

except for the addition of Np to one treatment. The N source for both

treatments was fertilizer grade crystaline $(NH_4)_2SO_4$ and was applied

at 90 kg N/ha. One treatment received no Np while the other was

treated with Np at the rate of 140 g/ha (0.0625 ppm soil basis or

0.156% based on N). A measured amount of $(NH_4)_2SO_4$ was placed

in a twin-shell blender and the appropriate amount of Np (22.2% active

Lot no. WP 10314-1) added, mixed for 30 minutes, and placed in high

Table 1. Chemical and physical characteristics[z] of the soil from
the 1975 field experiment.

Texture (feel)	Sandy loam
pH (paste)	7.9
$CaCO_3$ (%)	0.9
Total soluble salts (mmhos/cm)	1.1
Organic matter (%)	1.6
NO_3-N (ppm)	40
Available P (ppm)	28
Available K (ppm)	380
DTPA Ext. Zn (ppm)	0.8
DTPA Ext. Fe (ppm)	6.0

[z]$CaCO_3$ - acid-neutralization determination
  Total soluble salts - filtered extract from saturated soil
          paste was measured for conductivity
  Organic matter - potassium dichromate colorimetric
          determination
  NO_3-N - phenoldisulfonic determination
  Available P - ascorbic acid determination
  Available K - ammonium acetate extraction and flame
          photometry
  Zn and Fe - DTPA extraction and atomic absorption
          spectrophotometry

density polyethylene buckets, sealed, and stored at $4^\circ C$ until application.

Seed was planted and treatments applied on June 12. Seed beds 1 m wide were formed prior to planting. Treatment plots were 4 rows wide and 7.5 m long. Treatments were banded 7.5-10 cm below and 5 cm to the irrigation furrow side of the seed row. The spinach cultivar was 'Bloomsdale Longstanding'. Seed was treated with Captan to prevent possible damping off. The two treatments selected were replicated six times and randomly assigned within blocks with 10 other treatments with varying N and Np rates.

Gated pipe was placed at one end of the field with irrigation set for every furrow. Plots were irrigated approximately every 7 to 10 days depending upon soil moisture observations. Spinach was cultivated once, hand weeded twice and required no pesticide applications. Visual observations regarding any treatment differences were recorded throughout the experiment.

Yield data and tissue and soil samples were taken from the middle 2 rows of each 4-row plot to prevent possible error due to adjacent treatments. A 1.5 m buffer section was designated at both ends of each 7.5 m plot while the middle 4.5 m were used for sampling. One meter sections were harvested from each plot when the plants were at market maturity, 56 days after planting.

Spinach plants were cut just below the crown and the total weight and number of plants were recorded for each plot. Tissue was sealed

in plastic bags, packed with ice in polystyrene coolers and stored

at $4^{\circ}C$ for transportation to Fort Collins. Leaf red light absorbance

(proportional to green color) and carotene content measurements were

made in Fort Collins following the procedure outlined under the heading

"Both Experiments" page 36.

Plants were dried in a forced draft oven at $70^{\circ}C$ for 48 hours,

then ground with a stainless steel Wiley mill through a 40-mesh stain-

less steel screen. Tissue from 1 m plot sections was harvested from

the two middle rows of each 4-row plot and combined to form one

sample. Tissue was analyzed by Ag-Consultant Laboratory, Brighton,

Colorado, for Fe which was by atomic absorption spectrophotometry.

NO_3 concentration of tissues was not measured in the field experiment.

Soil samples were collected from each plot 42 days from planting.

Five soil cores, 0 to 30 cm, were taken at random from each of the

middle 2 rows of each plot and combined. Soil samples were spread

on paper in the greenhouse the day following sampling and air-dried

for 48 hours. Soil samples were ground by a ball mill to a mesh size

of 10 and stored for analysis. Soil samples were analyzed for pH on

a soil paste by a glass calomel electrode system (20) and NH_4-N and

NO_3-N determined by Orion specific ion electrodes and meter (6, 30,

104). A paired "t" test was used to determine treatment differences

(124).

Growth Chamber Experiment 1976

'Bloomsdale Longstanding' spinach was grown in three Percival model MB-60B growth chambers. A completely randomized design including four N treatments was employed in each chamber. Treatments within chambers were represented by seven pots per treatment making a total of 28 pots per chamber. All 28 pots were completely randomized within each chamber every two weeks to minimize within chamber variability. Containers used were number 10 steel cans filled with 2 kg of soil mix on a dry weight basis and lined with 6 mil polyethylene bags to provide a nonleaching system.

An unsterilized soil mix of two parts sandy loam plus one part perlite by volume was used. Soil NH_4-N, NO_3-N, and pH was determined at the start of the experiment following the same procedures described for the field experiment. Soil chemical and physical characteristics are shown in Table 2.

Eight seeds per pot were planted and all treatments were watered to field capacity on April 16. The soil surface was kept moist until after emergence. Subsequent thinning, after 15 days, left two uniform plants per pot. Container weights were monitored to maintain soil moisture at 0.3 bar. The soil perlite mixture contained 21% moisture at 0.3 bar as determined by a previously constructed moisture tension curve. Watering volumes were uniform for all treatments.

Table 2. Chemical and physical characteristics[z] of the soil mix used in the growth chamber experiment.

Texture (feel)	Sandy loam
pH (paste)	7.1
$CaCO_3$ (%)	0
Total soluble salts (mmhos/cm)	0.5
Organic matter (%)	0.8
NH_4-N (ppm)	12
NO_3-N (ppm)	29
Total N (%)	0.052
Available P (ppm)	13
Available K (ppm)	425
DTPA Ext. Zn (ppm)	0.19
DTPA Ext. Fe (ppm)	2.0
Cation Exchange Capacity	9.2

[z]$CaCO_3$ - acid-neutralization determination

Total soluble salts - filtered extract from saturated soil paste was measured for conductivity

Organic matter - potassium dichromate colorimetric determination

NH_4-N - specific ion electrode

NO_3-N - phenoldisulfonic determination

Percent total N - Kjeldahl

Available P - ascorbic acid determination

Available K - ammonium acetate extraction and flame photometry

Zn and Fe - DTPA extraction and atomic absorption spectrophotometry

Cation Exchange Capacity - NaOAc saturation and flame photometry

A 12 hour photoperiod was maintained. Light intensity ranged from 2800 to 3700 foot-candles within each chamber. A light period temperature of $24^{\circ}\pm 2^{\circ}C$ and a dark period temperature of $16^{\circ}\pm 2^{\circ}C$ was maintained. Visual observations of differences among treatments were taken throughout the experiment.

Treatments were constructed to provide differing ratios of NH_4 to NO_3 in the soil using Np to hold applied NH_4 in NH_4 form by suppressing nitrification throughout the experiment. Each treatment had 2 control pots in which no plants were grown. Moisture content and other parameters were maintained as for pots containing plants. The control pots were designed to give an estimate of nitrification activity in the absence of plant uptake influence.

All treatments consisted of 2 kg (dry weight) soil-perlite mix, 333 mg P as $CaHPO_4$, and 400 mg N in differing forms and NH_4/NO_3 ratios as follows:

Treatment 1: 400 mg N/2 kg soil mix as $(NH_4)_2SO_4$ (reagent grade) with 3 mg active Np/2 kg soil mix (1.5 ppm dry soil basis and 0.75% of the applied N) designed to hold all soil N in NH_4 form for the duration of the experiment. The appropriate amount of Np (38.3% active, Lot no. M 4100) was applied directly to the crystaline $(NH_4)_2SO_4$ and mixed in a twin-shell blender for 2 hours and stored in a sealed glass jar at $4^{\circ}C$ until blending with the soil mix.

Treatment 2: 400 mg N/pot in NO_3 form using $Ca(NO_3)_2$.

Treatment 3: Total N application divided into 100 mg N/pot from $(NH_4)_2SO_4$ with 3 mg Np/pot and 300 mg N/pot from $Ca(NO_3)_2$ to induce an NH_4/NO_3 ratio of 0.33. Application of Np to $(NH_4)_2SO_4$ was accomplished as in treatment 1 above.

Treatment 4: 400 mg N/pot applied as $(NH_4)_2SO_4$ without Np. The indigenous soil mix nitrifiers would convert this NH_4 to NO_3 at an unknown rate assuming environmental conditions conducive to nitrification.

All treatment fertilizer applications were weighed into charges for 2 kg dry soil mix, placed in glass vials, and sealed until soil incorporation. After moisture content of the soil mix was determined, an amount was weighed to contain 2 kg soil mix on a dry weight basis and placed in a twin-shell blender. After the appropriate fertilizer charges were added, the mixture was allowed to blend for 1 minute and then emptied into a plastic lined pot. Seed was then sown and water applied as described above.

Tissue from all treatments was harvested on June 7, 53 days from planting. Six leaf red light absorbance measurements from different leaves were obtained from each pot just prior to harvest using the equipment and procedure described under the heading "Both Experiments" page 36. Spinach plants were severed at the soil surface and weighed individually followed by a leaf count including all leaves

showing a visable petiole, standing upright (off the soil), and having at least some part of the lamina showing green color. Some senescent leaves were not included in any measurements. Plants were individually placed in paper bags and dried in a forced draft oven for 30 hours at $70^{\circ}C$. Dry plant tops were weighed then ground with a stainless steel Wiley mill through a 40-mesh stainless steel screen. Dried, ground tissue was stored in a freezer at $-15^{\circ}C$ until analysis for carotene and Fe was performed. Tissue samples were analyzed at the Soil Testing Laboratory at Colorado State University for Fe by atomic absorption spectrophotometry.

Soil samples were obtained by combining four soil cores from each of seven pots making up a treatment replicate. After thorough mixing of the composite, subsamples were taken for determination of total N, NH_4-N, NO_3-N, and pH, using the methods described for the field experiment. Analyses were performed by the Soil Testing Laboratory, Colorado State University. Establishment of soil NH_4-N/ NO_3-N ratios for each treatment followed. A one-way analysis of variance with replication was used as the statistical analysis procedure (124).

Both Experiments

A "chlorophyll meter" developed by Ennes and Associates of Riverside, California, was used to quantify green leaf color. The

meter responds to a sensing unit containing a light source and a photo-cell that measures red light (610-700 nm) reflected from the leaf surface. The meter scale is labeled absorbance though an accurate absorbance measuring system would have to account for light trans-mitted by a leaf which this instrument does not. An absorbance scale has the advantage of giving readings that vary directly with the log of chlorophyll concentration and hence green color (147). The lower the level of reflected light, or the greater absorbance, the more chloro-phyll the tissue contains and the darker green is the leaf. Henceforth, green color measurement will mean leaf red light reflectance measure-ment using the chlorophyll meter described above. Following is the procedure developed to quantify spinach green leaf color:

1. Tissue samples from the field experiment were kept in a cooler prior to color measurement to prevent wilting. Color measure-ment for growth chamber spinach was performed just before harvest while leaves were still on the plants.

2. Ten recently matured leaves were randomly chosen from the field sample comprised of all the leaves from one treatment repli-cate. Six measurements were taken on six recently matured leaves in each pot from the growth chamber experiment.

3. The upper surface of each leaf was used to measure the level of absorbed light.

4. For standardization, an area in the middle of the laminae, just to the right of the midrib (with the petiole towards the operator) was used for measurement.

5. The sensing unit of the reflectance meter was placed on the smoothest portion of the area chosen.

6. A blank white index card was used for the zero reading. The meter was readjusted to a zero setting after every 30 measurements.

Tissue from the field experiment was analyzed for carotene by replicate. Six replicates provided two 1 g aliquots each making a total of 12 analyses for each of the two treatments in the field experiment. Dry tissue from seven pots (2 plants/pot) of each treatment replicate in the growth chamber experiment was combined, mixed and subsamples were taken therefrom. This provided 11 and 23 degrees of freedom for carotene measurement for the field and growth chamber experiments respectively. Two 1 g aliquots of dry tissue were taken from each of three treatment replicates of four treatments making a total of 24 analyses for the growth chamber experiment.

The procedure followed for carotene determination in dried spinach leaves was the cold saponification method developed by Livingston (84). An * in the following procedure will indicate modification of the procedure as proposed by Livingston.

Grind all samples to pass no. 40 sieve. Weigh 1.0000 g dried spinach * into 100 ml volumetric flask. Pipet 30 ml hexane-acetone (7+3) into flask and add 1" * Teflon-covered stirring bar; suspend flask $1\frac{1}{2}$" above magnetic stirrer. While solution is smoothly stirring, pipet 2 ml 40% methanolic KOH into flask. Stopper with rubber stopper and continue stirring $\frac{1}{2}$ hour. Pipet 0.5 ml deionized water into flask, and continue stirring 1 minute. Remove flask from stirrer and retrieve stirring bar with magnetic stirring bar "retriever," rinse bar into flask with several ml hexane. Dilute solution to volume with hexane, stopper, mix by inverting 5 times, and let settle in dark $\frac{1}{2}$ hour.

Column chromatography follows: Place glass column (1.2 cm x 30 cm) fitted with rubber stopper on clean vacuum filter flask; place absorbant cotton plug in bottom of column, apply vacuum, and add adsorbant * (mix in mechanical blender 1-2 hours 1 + 1 w/w silica gel G, according to Stahl, Brinkman Instruments, Inc., and diatomaceous earth, Hyflo Super-cel) to ca 7 cm height when packing firmly with a glass rod. Add 2 cm layer of anhydrous Na_2SO_4 above adsorbant and press firmly.

Pipet 10 ml extract (20 ml extract was used for field experiment tissue due to low carotene content) onto column and adjust vacuum for flow of 2-3 drops/second. Add carotene eluant hexane-acetone (9+1) as last of solution enters adsorbant and continue until carotene band

is eluted into flask. The first band through the column is carotene. Release vacuum, remove column apparatus, and carefully pour flask contents into 50 ml volumetric flask *, rinsing vacuum flask with several ml eluant. Bring to volume with eluant and mix, bring to room temperature, and promptly determine absorbance at 436 nm. A Spectronic 20 spectrophotometer was used.

Calculations of mg carotene per kg fresh tissue were based on the mean of two separate aliquot extractions. Absorbance for known concentrations of carotene were determined using a mixture of 90% beta- and 10% alpha-carotene dissolved in hexane-acetone (9+1). Sample A readings were compared with known concentration values and equations shown in appendix figure 1 were used to calculate mg carotene/kg fresh spinach. Fresh spinach tissue was assumed to contain 10% dry matter. The coefficient of variability for the carotene analysis method used in this study was 1.02%. Regression analyses to determine correlation coefficients were performed on carotene as a function of green color, leaf NO_3-N, soil NH_4-N, NO_3-N, pH, and spinach yield. Total carotene in these experiments was considered a measure of provitamin A content.

RESULTS

Field Experiment 1975

Examination of the results of the two treatments selected from the 1975 field experiment reveals that they are representative of the observations noted in other treatments with different N and Np application rates, and harvest times (113). The two treatments were chosen to determine a possible difference in carotene content since the two treatments demonstrated the largest difference in green color in the entire experiment. It was also of interest to determine if the lowest Np rate (140 g/ha) could induce significant increases in provitamin A content. The overall observations of the field experiment showed that Np applications increased yield, tissue green color, and Fe (113). Comparison of the two treatments chosen from the field experiment follows the soil and plant data appearing in Tables 3 and 4.

Soil NH_4 and NO_3-N were higher for the Np treatment than for the treatment receiving no Np shown in Table 3. This indicates a higher N availability with Np. The found NH_4/NO_3-N ratios for the two treatments support the hypothesis that spinach utilizes NH_4 or NO_3 (113,127). The soil NH_4/NO_3-N ratio was higher in the Np treatment indicating the soil N was in predominantly NH_4 form at the time of sampling. The soil NH_4/NO_3 ratio has been shown to change

Table 3. Results of soil tests with NH_4-N/NO_3-N ratios for treatments selected from the 1975 field experiment. Soil samples were taken 42 days from planting for the "found" N.

| Treatment | | | Applied plus residual | | | Found | | |
N source (90 kg/ha)	Nitrapyrin (g/ha)	pH	NH_4-N (ppm)	NO_3-N (ppm)	NH_4/NO_3 (ratio)	NH_4-N (ppm)	NO_3-N (ppm)	NH_4/NO_3 (ratio)
$(NH_4)_2SO_4$	0	7.9	160	17	9.4	11	12	0.92
$(NH_4)_2SO_4$	140	7.9	160	17	9.4	44**	12	3.67

**significant at the 1% level within the column.

Table 4. Plant tissue data from 1975 field experiment showing influence of nitrapyrin.

Treatment		Tissue			
N source (90 kg/ha)	Nitrapyrin (g/ha)	Yield (MT/ha)	Fe (ppm)	Color (absorbance)	Carotene in fresh tissue (mg/kg)
$(NH_4)_2SO_4$	0	14.3	395	0.75	35.2 ± 1.8^z
$(NH_4)_2SO_4$	140	17.8^{**}	426^*	0.80^{**}	$40.5 \pm 0.9^{**}$

[z] Mean and standard error, N=12.
[*] significant at the 5% level within the column.
[**] significant at the 1% level within the column.

with time (114) and it is expected that these ratios did not exist throughout the experiment. Higher soil NO_3-N was expected for the treatment without Np as was noted in other treatments (113) due to nitrification though irrigation with subsequent leaching may be responsible for lowering soil NO_3 levels in treatments where NO_3 was more abundant.

Original soil pH was 7.9 and varied between 7.9 and 8.1 with no pH differences between treatments. The field soil had a high buffering capacity.

A significant increase in yield over the no Np treatment was noted for the Np treatment and is consistent with yield increases in other similar treatments (113). A paired "t" test (124) with 11 degrees of freedom on carotene content data showed that the 5.3 mg/kg fresh tissue difference in average carotene content was significant (P< 0.05) assuming fresh spinach tissue contained 10% dry matter. Tissue from the Np treatment, with 40.5 mg/kg carotene on a fresh weight basis, contained 15% more carotene than the no Np treatment with 35.2 mg/kg. Using both yield and carotene increases on a fresh weight basis to calculate a carotene yield (kg carotene/ha), a 43% increase is noted for the Np treatment when compared to the no Np treatment. This demonstrates that a significant increase in carotene content may be expected when a significant difference in green color is noted. Regression analysis for carotene as a function of green

color, illustrated in Figure 1, showed a close relationship between carotene and green leaf color of spinach. The model suggests that in the deep green color region, carotene content does not increase. Leaf concentration of Fe was increased with the Np treatment.

Growth Chamber Experiment

Soil analyses reflect suppressed nitrification in treatments containing Np in the growth chamber. As expected, fertilizer applied as NH_4 remained in that form where Np was employed in the growth chamber nonleaching system. Treatments receiving all or mostly NO_3 showed highest soil NO_3-N levels at the end of the experiment. A compilation of soil data appears in Table 5. Ammonium fertilized treatment 1 with 1.5 ppm Np showed very little NO_3 at the end of the experiment indicating substantial nitrification suppression compared to NH_4 fertilized treatment 4, receiving no Np. Nitrification did occur in pots not treated with Np. This is evidenced by no plants control pots, fertilized with NH_4, showing a decrease in NH_4-N at the termination of the experiment. Nitrification was suppressed almost totally in the 0.33 NH_4/NO_3 ratio treatment 3 with Np as shown by the small difference between applied and found NH_4/NO_3-N ratio in the no plants control pot. Final soil tests showed the amount and form of N left in the soil as a combined effect of the applied treatments and plant uptake.
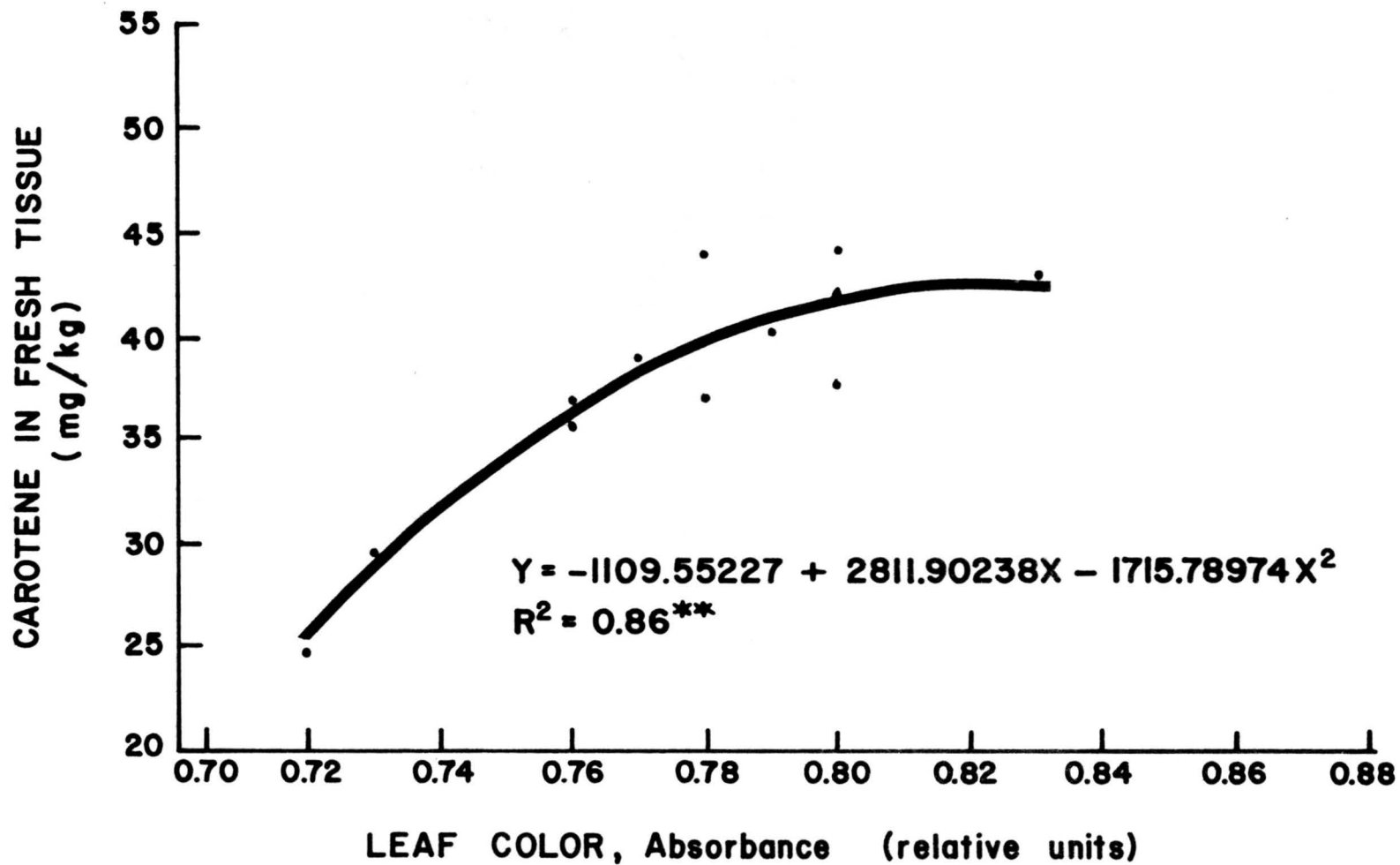
Figure 1. Spinach carotene content as a function of green leaf color from the field experiment.

Table 5. Applied plus residual and found soil NH_4-N/NO_3-N ratios and average pH for the growth chamber experiment. All treatments involved 400 mg N/2 kg soil mix.

Num-ber	Treatment N source	Np (ppm)	pH	Applied N plus residual NH_4-N (ppm)	NO_3-N (ppm)	NH_4/NO_3 (ratio)	Found N NH_4-N (ppm)	NO_3-N (ppm)	NH_4/NO_3 (ratio)
1	+ $(NH_4)_2SO_4$	1.5	6.3	229	12	19.00	72	3	24.00
1	$(NH_4)_2SO_4$	1.5	7.1	229	12	19.00	180	16	11.25
2	+ $Ca(NO_3)_2$		7.4	29	212	0.14	8	86	0.09
2	$Ca(NO_3)_2$		7.1	29	212	0.14	8	192	0.04
3	+$(NH_4)_2SO_4$+$Ca(NO_3)_2$	1.5	7.2	79	162	0.14	17	65	0.27
3	$(NH_4)_2SO_4$+$Ca(NO_3)_2$	1.5	7.2	79	162	0.14	55	134	0.41
4	+ $(NH_4)_2SO_4$		5.6	229	12	19.00	48	20	2.44
4	$(NH_4)_2SO_4$		5.4	229	12	19.00	86	98	0.88
LSD 5%			0.3[z]				15	23	2.53

[z]LSDs separate means of treatments which include plants (+).

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Examination of the NH_4/NO_3-N ratio for each treatment also indicated nitrification was suppressed where Np was applied. A compilation of the average ratios for all treatments, with and without plant influence, appears in Table 5. The NH_4 treatment 1, with Np including plants, showed an increase in NH_4/NO_3-N ratio during the experiment. Soil pH was markedly influenced by treatment as shown in Table 5. The pH of treatments 2 and 3, in which little or no nitrification took place, were not significantly different and were close to neutral. The presence or absence of plants had little effect in these two treatments. Nitrification suppression in treatment 1 held soil N as NH_4. Without plants, the pH of treatment 1 did not differ from treatments 2 and 3. With plants, treatment 1 showed a lower pH than treatments having at least some NO_3. Nitrification lowered the soil pH of treatment 4 about 1.5 pH units since it was not inhibited and applied NH_4 fertilizer provided the substrate. The presence of plants did not seem to change the relationships among treatments 2, 3, and 4 with regard to soil pH.

Spinach Yield

Yield parameters (Table 6) indicate that treatment 1, NH_4 plus Np showed the least average fresh and dry weight and number of leaves. Dark green leaf color, sensitivity to water stress, and brownish colored roots were also noted for this treatment. Plants receiving at least some NO_3 fertilizer showed maximum yield and

Table 6. Growth chamber spinach plant yield and tissue analysis data. All treatments involved 400 mg N/2 kg soil mix.

	Treatment		Yield		Tissue				
Number	N source	Np	Leaves/ plant	Fresh weight/ plant	N	Fe	NO_3-N	Green color (absorbance)	Carotene in fresh tissue
		(ppm)		(g)	(%)	(ppm)	(ppm)		(mg/kg)
1	$(NH_4)_2SO_4$	1.5	15.2	9.0	6.6	383	617	0.867	75.1±3.1 [z]
2	$Ca(NO_3)_2$		18.6	16.8	6.3	322	10166	0.741	52.0±3.0
3	$(NH_4)_2SO_4+Ca(NO_3)_2$	1.5	19.5	17.6	6.5	340	10400	0.778	64.9±2.5
4	$(NH_4)_2SO_4$		18.4	14.9	6.6	282	7700	0.785	78.5±2.0
LSD 5%			1.4 [y]	1.7	NS	NS	3848	0.017	4.2

[z] Mean and standard error, N=6.
[y] LSDs separate means of treatments within a column.

were not different from the plants in treatment 4 receiving NO_3 through nitrification. The NO_3 treatment 2 was not significantly different (P< 0.05) from the 0.33 NH_4/NO_3 ratio or NH_4 treatment 4 without Np. The protected, NH_4 treatment showed a significantly lower (P< 0.05) fresh weight than all other treatments. Dry weight comparisons demonstrated the same relationships. The number of leaves at maturity did not differ among treatments except that plants in the protected NH_4 treatment showed fewer leaves and hence, were not as mature as plants in other treatments due perhaps to ammonium toxicity.

Spinach Leaf Color

The darkest green leaf color was noted in the NH_4 fertilized treatment with Np, which grew with essentially all NH_4 nutrition. This treatment had significantly greener color (P< 0.05) than any other treatment. The color of treatment 1 leaf tissue was visibly darker 45 days from planting. Color differences among other treatments were not so obvious but were noted using a reflectance meter as previously described. Reflectance meter measurement showed the 0.33 NH_4/NO_3 ratio and the unprotected NH_4 treatments 3 and 4 respectively did not differ though both exhibited darker green leaves than the NO_3 treatment 2 (P< 0.05) which exhibited the lightest green leaf color in the experiment. Leaf Fe concentration measurements

shown in Table 6 proved to be too variable to establish significant differences ($P < 0.05$).

Several regression and correlation analyses were performed using leaf color as a function of dry and fresh weights, number of leaves, soil NH_4-N, NO_3-N, pH, and plant NO_3-N, Fe, and N content. Coefficients of determination with corresponding degrees of freedom are found in appendix table 1. Graphs of leaf color as a function of soil and leaf NO_3-N appear in Figures 2 and 3 respectively and show leaf color to be negatively related to soil and leaf NO_3-N. Soil and leaf NO_3-N were the only parameters which correlated significantly ($P < 0.05$) with green leaf color of spinach in the growth chamber experiment. Soil NH_4-N data in the growth chamber experiment was too variable, due possibly to nitrification in the unprotected NH_4 treatment, to establish a definite relation with leaf color though a tendency was noted for darker leaf color to occur with increasing concentrations of NH_4 in the soil.

Spinach Leaf Nitrate

Leaf NO_3-N was significantly reduced ($P < 0.05$) with NH_4 nutrition as shown in Table 6. The other three treatments did not differ significantly in NO_3 content though the lowest mean value of the three was the NH_4 fertilized treatment without Np. These observations follow a pattern indicating increased tissue NO_3-N with increasing soil NO_3 availability (98). A positive correlation coefficient of 0.85
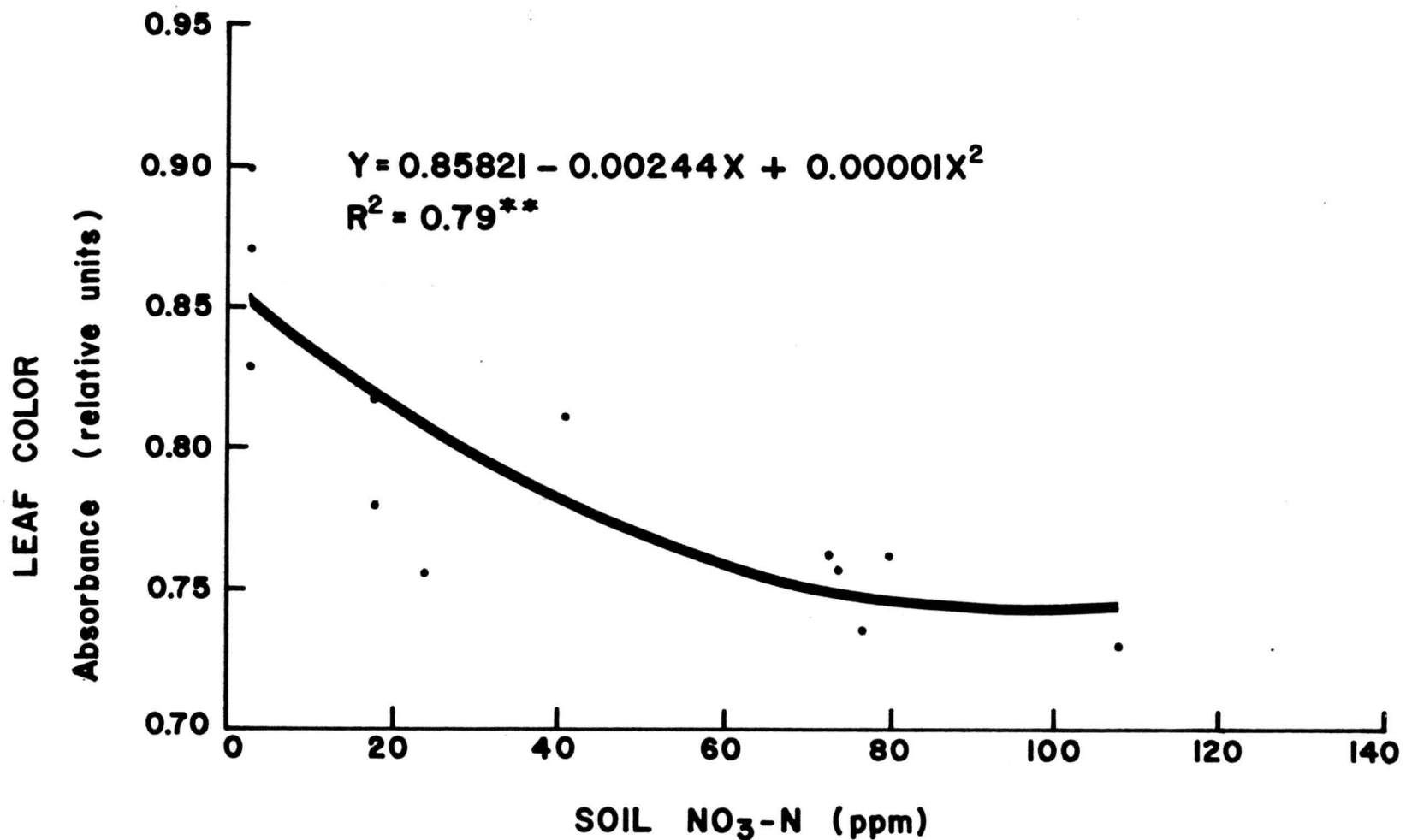
Figure 2. Spinach leaf green color as a function of soil NO_3-N. Data obtained from the growth chamber experiment. The more appropriate regression of leaf color on soil NH_4 was positive, however, the relationship was not significant at the 5% level of probability.

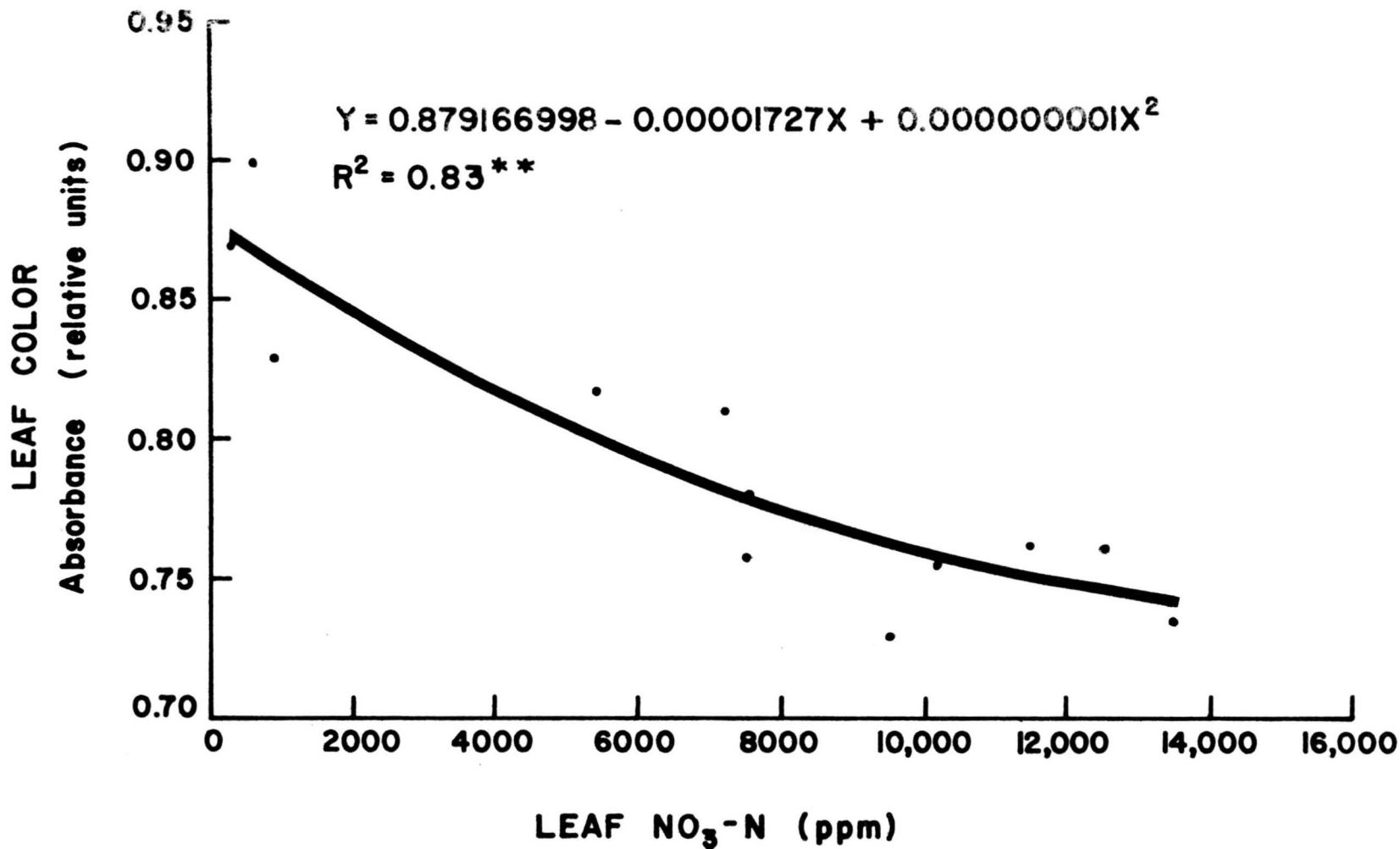Figure 3. Green leaf color as a function of leaf NO_3-N concentration for spinach grown in the growth chamber. This inverse relationship was obtained because treatments causing the darkest green color supplied the highest soil NH_4/NO_3 ratio.

was determined for NO_3 content of leaf tissue and soil NO_3-N. Treatment 2, fertilized with NO_3, showed the lowest tissue N concentration. Measurements were too variable to establish significant differences. Treatments receiving all applied N as NH_4 had statistically equal and highest mean N content of tissue.

Spinach Carotene Content

Ammonium nutrition significantly increased the carotene content of spinach as shown in Table 6. The highest carotene content was 78.5 mg/kg fresh tissue measured in the unprotected NH_4 treatment 4. Addition of Np to this treatment produced 75.1 mg/kg carotene and was not significantly different from the protected NH_4 treatment 1. Nitrapyrin did not have a significant effect on carotene content of spinach in the growth chamber experiment though a preponderance of soil NH_4 produced tissue with the greatest carotene content. The 0.33 NH_4/NO_3 ratio treatment 3 produced 64.9 mg/kg carotene, significantly lower than NH_4 treatments 1 and 4 and higher than the NO_3 treatment 2. Nitrate fertilized plants produced an average carotene content of 52.2 mg/kg, the lowest of the treatments in this experiment.

Regression and correlation analyses produced linear coefficients of determination, appearing in appendix table 1, for carotene content as a function of fresh and dry weight, number of leaves, soil NH_4-N, NO_3-N, pH, and N content, leaf color, Fe, NO_3-N, and total N uptake. No significant correlations were noted due to small sample

size and variability associated with the various parameters in the growth chamber experiment. There was a tendency for carotene content of leaves to be negatively related to soil NO_3-N and positively related to soil NH_4-N. Further investigation is needed in this area with larger sample sizes and more controlled soil NH_4 variation.

DISCUSSION AND CONCLUSION

Soil Nitrogen Form and pH

Treatments applied in both experiments effectively manipulated the NH_4/NO_3 ratio of the soil. Factors noted as influencing this ratio during the experiments were nitrification and plant uptake. Perhaps denitrification was also influencing this ratio. Where nitrification was inhibited, or no NH_4 fertilizer was applied, changes in the NH_4/NO_3 ratio may be attributed to plant uptake or denitrification. Nitrate leaching in the field experiment undoubtedly had an influence on the soil NH_4/NO_3 ratio. Unaltered nitrification drastically lowered the NH_4/NO_3 ratio in NH_4 fertilized treatments. Suppressed nitrification of NH_4 fertilizer caused an increase in NH_4/NO_3 ratio due to preferential plant uptake of available NO_3. With plants absent, the protected NH_4 treatment in the growth chamber experiment caused a decrease in the NH_4/NO_3 ratio and was perhaps due to a small amount of nitrification [116]. The protected NH_4 treatment had a lower pH than treatments receiving at least some NO_3 due to H ions given off during NH_4 uptake and assimilation into organic forms [65,76]. Sharp reduction in soil pH also indicates active nitrification [20] in treatments where Np was not applied.

Comparison of planted vs. unplanted pots of a given treatment demonstrates that soil pH in the growth chamber experiment was affected by one or both of two processes; 1) nitrification and 2) NH_4 uptake and assimilation, by spinach plants (65). Ammonium fertilized spinach with Np showed a drop in soil pH in the presence of plants and is attributable to NH_4 uptake and assimilation. Treatments 2 and 3 having little or no NH_4 or nitrification processes showed no difference between planted and unplanted pots. Treatment 4 had the greatest pH drop and may be attributed to both NH_4 uptake and assimilation along with unlimited nitrification.

Accounting for all soil and plant nitrogen in the growth chamber experiment was impossible due to small sample sizes and plant uptake variability. The reduced N uptake of spinach fertilized with NH_4 plus Np may have been due to reduced dry matter accumulation as a result of ammonium toxicity (98). Dry weight is calculated as 10% of fresh weight shown in Table 6. A decrease in plant N may have been further exaggerated by senescent or dead leaves in the protected NH_4 treatment not included in dry weight measurements.

A tendency of N content of spinach tissue to decrease with NO_3 nutrition was noted (98). Treatment 2 fertilized with NO_3 showed the lowest tissue N content. Measurements were too variable to establish significant differences though NH_4 fertilized spinach had higher mean N contents than NO_3 fertilized spinach. This supports conclusions

reached by Mills (98) that N concentration of spinach leaves increases with each increment of $(NH_4)_2SO_4$ fertilizer up to 800 mg/pot while increases in N content stopped at 400 mg/pot when KNO_3 was the N source, presumably increments beyond 400 mg/pot exceeded amount optimum for plant growth.

Based on data presented in Table 6 and assuming dry matter is 10% of fresh weight, average N uptake for treatment 1 was 143 mg/pot. Uptake of N by other treatments did not differ significantly and these healthy spinach plants took up 200 mg N/pot during the experiment. The presence of plants decreased soil NH_4 and NO_3-N in all treatments (Table 5). Losses in soil N in treatments where plants were not grown during the experiment may be attributable to denitrification. Reduced water uptake due to ammonium toxicity affected plants may have accelerated denitrification losses in treatment 1.

Spinach Yield

Yield was adversely affected under suppressed nitrification conditions when NH_4 was the only source of N available to spinach plants. Evidence of ammonium toxicity including reduced yield, stunting, sensitivity to water stress, dark colored roots, and dark green leaves were noted in treatments with Np protected NH_4 as the sole N source (7, 98, 113, 119, 127). Spinach therefore requires at least some NO_3 during growth to achieve maximum yields and/or to avoid ammonium

toxicity (113). Optimum yields may be obtained by maintaining a 0.33 NH_4/NO_3 ratio throughout the experiment incorporating Np at 1.5 ppm to suppress nitrification of NH_4 to NO_3 (114). This ratio is approached with time by unaltered nitrification of applied $(NH_4)_2SO_4$ fertilizer as shown in the growth chamber experiment. Maximum yields were also attainable with NH_4 or NO_3 sources in the growth chamber.

The use of Np in high buffering capacity field soils significantly increased yield and did not cause ammonium toxicity to spinach plants indicating that ammonium toxicity may be avoided by maintenance of high pH (9, 113, 120, 149). The growth chamber soil showed poor buffering capacity and hence contributed to conditions favoring ammonium toxicity (65), however, NH_4-N concentrations less than 400 mg/2 kg soil mix and/or less than 1.5 ppm Np should alleviate NH_4 toxicity symptoms. Use of protected NH_4 fertilizers, especially with furrow irrigation, will reduce leaching losses of N and increase N fertilizer efficiency especially in field soils with high buffering capacities.

Spinach Leaf Color

Predominance of NH_4 or NO_3 in the soil had a pronounced effect on spinach leaf color in both experiments. Examination of all of the correlations of the various parameters and green leaf color indicates that leaf and soil NO_3 concentrations are inversely related to spinach

green leaf color. This is supported by the NO_3 fertilized treatment 2 showing the lightest green leaf color. The data presented here cannot be used to evaluate the theories of Machold (87), indicating an increase in green color under ammonium nutrition may be due to an increase in iron utilization within the plant. Further research is needed in this area. Fe uptake was increased significantly in the field experiment (113) as shown in Table 4, however, a very poor correlation between leaf Fe and leaf color was noted. Variability associated with leaf Fe concentration measurement precluded establishment of significant differences in the growth chamber experiment though a trend was noted for Fe uptake to increase with NH_4 nutrition.

The NH_4 fertilized treatment 1 with Np produced the darkest green leaf color though accompanying toxicity symptoms result in unacceptable market quality. Toxicity symptoms may be eliminated under field conditions with high buffering capacity soils therefore NH_4 nutrition may be optimum for field spinach production in alkaline soils. Spinach production under nonleaching conditions would be optimized using unprotected NH_4 or a NH_4/NO_3 ratio of 0.33 maintained with 1.5 ppm Np.

Spinach Leaf Nitrate

Spinach leaf NO_3 is closely related to soil NO_3 concentration (96). Manipulation of soil N fertilizers to include a minimum of NO_3

is a realistic solution to plant NO_3 accumulation problems. Nitrapyrin application will retard conversion of applied NH_4 to NO_3, further reducing spinach leaf NO_3 content and hence the danger to human health and environment.

Spinach Carotene Content

Carotene content of spinach leaves is significantly influenced by soil N manipulations in this and other studies (16, 31, 46). The dominance of NH_4 in the soil increased carotene content by 18% above the 0.33 NH_4/NO_3 ratio treatment 3 and 43% above the NO_3 treatment 2. Application of nitrapyrin per se did not influence carotene content in the growth chamber experiment. A small difference in absorbance readings for leaf color measurements corresponded to large differences in carotene content as was true in a qualitative color study and were attributable to suppressed nitrification by Np in the field. Though leaf color measurements were highly variable as in another study (110), a conservative estimate of carotene content increase with a 0.05 increase in absorbance varies from 13 to 50%. Maintenance of soil N as NH_4 will produce significant increases in carotene content of spinach. A definite relationship between green color and leaf carotene has been clearly demonstrated for the first time. This work illustrates that by proper manipulation of the soil NH_4/NO_3 ratio using very low levels of nitrapyrin, spinach yield and provitamin A content can be increased substantially.

SUMMARY

The nitrification suppressant, nitrapyrin was used with $(NH_4)_2SO_4$ to determine the effect of soil NH_4/NO_3 ratio on spinach yield, green color, and provitamin A in a field and growth chamber experiment. Spinach NO_3 was measured only in the growth chamber experiment. Significant yield increases using Np in the field as a coating on $(NH_4)_2SO_4$ fertilizer was attributed to increased soil N retention. Treatments applied in the growth chamber effectively manipulated the soil NH_4/NO_3 ratio and induced ammonium toxicity symptom expression in spinach fed only NH_4 protected from nitrification by Np. Yield differences did not occur among treatments receiving NO_3 through applied NO_3 fertilizer or nitrification of NH_4 fertilizer.

The green color of spinach leaves was significantly darkened by supplying plants with NH_4 during growth. Darkening of green color is most directly affected by soil NH_4 though dark green color may also be due to increased Fe availability in the soil and subsequent increased plant uptake of Fe. Green leaf color of spinach was negatively correlated with soil and leaf nitrate. Spinach leaf NO_3 concentration was positively correlated to soil NO_3 availability. Supplying N as NH_4, especially in conjunction with Np, was an effective means of lowering leaf NO_3 concentration in growth chamber grown spinach.

The carotene content of spinach leaves may be influenced drastically by soil N manipulations. A 15% increase in leaf provitamin A content was noted using Np compared to treatments receiving equal N fertilization without Np in the field. Using carotene and yield data, an increase in provitamin A yield per unit area of 43% was noted for the treatment supplied with Np coated $(NH_4)_2SO_4$ compared to $(NH_4)_2SO_4$ alone. When $(NH_4)_2SO_4$ was the sole N source in the growth chamber, maximum carotene production occurred though no significant difference between these treatments with or without Np was noted regarding carotene content. This was perhaps due to small sample size, ammonium toxicity, or slow nitrification of unprotected NH_4. Spinach grown under NH_4 nutrition showed a 43-50% higher carotene content compared to treatments fertilized only with NO_3 in the growth chamber.

Small differences in green leaf color, as detected using a reflectance meter, may correspond to large differences in carotene content of spinach. Increases in absorbance readings over the range of 0.74 to 0.79 may indicate increases in carotene content of 40 to 50% in spinach leaves. A highly significant coefficient of determination of 0.86 for carotene as a function of green leaf color indicates the two parameters are closely related. Development of a predictive model for spinach carotene content will require highly controlled experimental conditions with large sample sizes. Nitrapyrin would be a useful tool with which to manage soil N form in such studies. A definite

relationship between green color and leaf carotene has been clearly demonstrated for the first time. This study illustrates that by proper manipulation of the soil NH_4/NO_3 ratio by using very low levels of nitrapyrin, spinach yield and provitamin A content can be increased substantially.

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APPENDIX

Table 1. Results of linear correlation and regression analyses on spinach leaf color, NO_3, and carotene from the growth chamber experiment. Degrees of freedom for each relationship appear in parenthesis under the corresponding coefficient of determination.

	Plant								Soil		
	Carotene	Color	Fresh weight	Dry weight	Number of leaves	Fe	NO_3-N	N content	NH_4-N	NO_3-N	pH
Plant											
Carotene		0.41 (11)		0.16 (11)	0.73** (11)	0.02 (11)			0.69* (11)	0.69* (11)	0.62* (11)
Color			0.25* (83)	0.36** (83)	0.23* (83)	0.03 (11)	0.83** (11)	0.41 (11)	0.50 (11)	0.65* (11)	0.52 (11)
Leaf NO_3-N									0.51 (11)	0.85** (11)	

*Significant at the 5% level.
**Significant at the 1% level.

A 1 ppm (1 mg/kg) solution of 90% beta-carotene and 10% alpha-carotene dissolved in reagent grade hexane gave an absorbance reading of 0.1875.

$$\frac{\text{(sample absorbance) x 50 ml final solution x}}{\text{0.1875 x 1000 ml final solution x 10 ml}} = \frac{\text{mg carotene}}{\text{kg fresh tissue}}$$

100 ml extracting solution x 1 g dry tissue
extracting solution x 0.01 fresh tissue
(20 ml extracting solution
used for field tissue)

Consolidating the above equation, a working equation for 1 g dry growth chamber tissue is:

(sample absorbance) x 266.667 = mg carotene/kg fresh tissue

for 1 g dry field tissue:

(sample absorbance) x 133.333 = mg carotene/kg fresh tissue

Figure 1. Equations for carotene calculations for growth chamber and field experiments.