SOME PHYSIOLOGICAL AND PHYTOTOXIC EFFECTS OF FENITROTHION ON GERMINATION AND SEEDLING GROWTH OF PINUS STROBUS L.

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TABLE OF CONTENTS

RÉSUMÉ

On a stratifié des semences de pin blanc dans l'eau ou dans une émulsion aqueuse de fénitrothion. On a utilisé deux concentrations de fénitrothion: 10 p.p.m. et 1,000 p.p.m. On n'a observé d'augmentation de l'absorption d'eau et en protéines de base (histones) que dans les semences mises en présence de 1,000 p.p.m. de fénitrothion. Toutefois, on a observé une activité enzymatique réduite (des estérases et des déshydrogénases succiniques) mise en évidence par les techniques de localisation, surtout dans la l'hypocotyle de l'embryon, après exposition aux deux concentrations en fénitrothion. Pendent 10 jours de germination, il n'y a pas eu d'effet apparent sur les polysaccharides insolubles totaux ou sur le métabolisne de l'ADN. Les pourcentages et les taux de germination n'ont pas différé de façon significative dans le groupe cobaye par rapport au groupe témoin. Après cinq mois, les jeunes pousses de pin blanc exposés à l'insecticide ne souffraient d'aucun défaut de croissance ou d'aucun défaut morphogénique.

INTRODUCTION

In Canada, fenitrothion has been applied as an aircraft spray to large areas of coniferous and mixed forests (Fettes, 1968). Although this program has ameliorated serious spruce budworm damage, the impact on reforestation has not previously been assessed.

The present study was undertaken in order to provide such basic information. The forest seeds discussed in this report are the Eastern white Pine (Pinus strobus L.), and the effects of two concentrations of fenitrothion - one representing field dosage and the other a supra-optimal dosage - on germination characteristics and seedling growth were investigated.

MATERIALS AND METHODS

Storage of Seeds:

The seeds of Pinus strobus L. (the eastern white pine) were obtained in late September, 1972 from the Beggar Lake region of the Petawawa Forest Experimental Station, Chalk River, Ontario. The Chemical Control Research Institute (Environment Canada) had aerially sprayed a specified area within this region with fenitrothion in the spring of 1972 (2-4 ounces/acre). The seeds used in this study were gathered from non-sprayed (c) and sprayed areas (s).

Extraction of pine seeds from the closed cones was carried out by incubation at a constant temperature of 28° C for approximately 36 hours (Wang, 1973). The seeds were stored in tightly sealed glass

containers at 2° C until required for experimental procedures.

Conditions of Imbibition and Stratification of White Pine:

In parallel experiments, seeds (10 per petri dish) were exposed either to a non-limiting amount of distilled water (10 ml. per petri dish) or to 10 ml of a water-fenitrothion emulsion. The seeds were then chilled (stratified) and maintained at 10° C for 21 days (Wang, 1973). Following this treatment, they were placed in environmental growth chambers and germinated, in the dark, under a 12 hour diurnal temperature regime of 86° F and 68° F (U.S.D.A., 1949).

Insecticide Treatment:

White pine seeds were exposed to fenitrothion emulsion either during the 21 days stratification period or during the initial three days of germination. The two treatments were used in order to observe whether there were any apparent differences with respect to the period of contact.

Two concentrations of fenitrothion were employed, namely 10 ppm and 1000 ppm. Ten ppm approximates a field concentration. Only premium grade fenitrothion (Sumithion) was used and this was kindly supplied by the Sumitomo Chemical Company of Japan.

Seed Germination:

All seeds used in this study were routinely sterilized for 10 minutes in a 2% hypochlorite solution and then thoroughly washed. The seeds were examined daily with a dissecting microscope (50 x magnification). They were considered to have germinated when the radicle pierced through the seed coat. Germination percentages and germination rates were then determined for each treatment (Maguire, 1962).

Three hundred white pine seeds were exposed to each fenitrothion concentration during stratification. This represented 30 replicates of 10 seeds. Total seed germination and germination rates were also determined on one hundred white pine seeds exposed to each fenitrothion concentration only during the first three days of germination. Thus, there were 10 replicates of 10 seeds for each treatment. White pine seedlings were also planted in potting soil immediately following germination and placed in a growth room maintained at 20^{o} C in order to monitor possible morphological deviations.

Water Uptake:

Percent water uptake of white pine seeds during the stratification-fenitrothion exposure period and the subsequent two days of germination was followed. Weight homogeneity of each treatment group was rigorously monitored to within ± 2.5 mg. The study was repeated at six different time periods to provide data that could be subjected to statistical evaluation. At each specified time period the seeds were reweighed.

The petri dishes containing the seeds were kept on ice during the rapid weighing procedure in order to minimize sudden temperature alterations. Six replicates of 10 seeds were followed for each treatment group. Then seeds were carefully botted dry before being weighed. The percentage increase in fresh weight was then determined after 1, 3, 7, 11, 14, 18 and 21 days of stratification. Insecticide exposure was terminated following the 21 day stratification period and the seeds were transferred to fresh petri dishes containing a non limiting amount of distilled water and placed in environmental growth chambers. Further weight changes were monitored following 2 days of germination.

Oxygen Uptake:

The oxygen uptake of white pine seeds was determined by the Warburg technique. Ten comparable seeds (± 2.5 mg) were used for each treatment. Seeds from the control and fenitrothion 1000 ppm treated groups were removed from stratification at each of the following periods, namely 5, 10, 15, and 21 days. These seeds were then suspended in 1.0 ml of 0.01 M phosphate buffer at pH 6.9 in Warburg vessels. Each vessel centre well contained 0.3 ml of 20% KOH and a small filter paper wick (Kozlowski, 1959). The seeds were allowed to equilibrate for 12 hours at 30° C before determinations were made. The data for each series was replicated three times.

Histochemical Studies:

White pine embryos were dissected from the seed gametophyte tissue after 0, and 21 days of stratification and also following 6, 8 and 10 days germination. During the dissection procedure, small amounts of phosphate buffer were added in order to prevent the tissues from drying out.

A random sampling of embryos from each of the three treatments (control, 10 ppm and 1000 ppm fenitrothion exposed seeds) was obtained at each sampling date.

Tissue preparation:

The white pine embryos which were to be investigated by light microscopy were unfixed and dehydrated in a graded tertiary butyl alcohol-ethanol series for infiltration and embedding in paraffin. Ten micron serial sections were cut on a standard rotary microtome and adhered to clean glass slides with Haupt's adhesive (Jensen 1961). Following

the cytochemical localization procedures all sections were re-hydrated and mounted in Canada balsam.

For enzyme localization studies (succinic dehydrogenases and esterases) intact embryos were excised and placed in the appropriate reaction mixture (see below) then fixed in Carnoy's fluid (Purvis, 1964). Following this, the specimens were embedded in Spurr's plastic (Spurr, 1969) and photographed.

Localization:

The following metabolic parameters were monitored by localization techniques; insoluble carbohydrates, DNA and basic proteins (histones), succinic dehydrogenase and esterase.

(a) Insoluble carbohydrates (Periodic acid Schiffs): Parffin was removed and the sections brought to water through a graded xylol-ethanol series. They were then immediately placed in a 1% periodic acid solution for 10 minutes (Durzan, 1971). The material was then washed in running water for 10 minutes and placed in freshly prepared Schiff's reagent for 30 minutes (Jensen, 1962). Following this, the sections were rinsed in 2% sodium bisulfite for 2 minutes, dehydrated and mounted.

(b) DNA: Sections were stained for DNA by the conventional Feulgen method (Jensen, 1962). Prior to staining, the cells were hydrolyzed in NH_4Cl at 60^{o} C for precisely 35 minutes).

(c) Proteins high in basic amino acids: Paraffin was removed from the sections and brought to water. The slides were then placed for 15 minutes in a 10% trichloroacetic acid solution in a boiling water bath to remove the nucleic acids. Following this, they were immersed in three changes of 70% ethyl acohol. Sections were then stained for 25

minutes in a 0.1% aqueous Fast Green FCF solution at pH 8.1, adjusted with a minimum of NaOH. Following a 5 minute wash in distilled water, the sections were placed directly in 95% ethyl alcohol and dehydrated (Alfert and Geschwind, 1953). The reaction which is quite specific for histones also stains any protein with high amounts of basic amino acids and the resultant colour may be measured microspectrophotometrically to indicate amounts (Jensen, 1962).

(d) Succinic Dehydrogenases: Freshly dissected embryos were placed in a 0.05 M phosphate at pH 7.0 and then transferred into the reaction medium composed of 5 ml 0.067M phosphate buffer at pH 7.0; 5 ml. of 0.02M sodium succinate; 5 ml of 0.1% neotetrazolium chloride and 5 ml of distilled water. This solution was boiled and cooled immediately prior to use. The embryos remained in the reaction medium for 2 hours at 37^{o} C (Avers, 1958). Controls were heat killed and placed in a complete reaction mixture (Molnar, 1972). Bright purple coloration indicated sites of enzyme activity.

(e) Esterases (Indiogenic method). Freshly dissected embryos were placed in a substrate solution composed of 1.5 mg of 5-bromo-4-chloroindoxyl acetate in 0.1 ml of absolute ethyl alcohol, 1 ml solution of equal parts of 5×10^{-2} M potassium ferricyanide and 5×10^{-2} potassium ferrocyanide, and 0.1 ml of 1M calcium chloride, 5.0 ml of 2M sodium chloride, 2.0 ml of 0.1M tris buffer at pH 8.5 and distilled water to 10 ml. Controls were heat killed and placed in a complete reaction mixture (Holt, 1958). All embryos in solutions were incubated at 37^{o} C for 1.5 hours. Sites of general esterases activity were indicated by a bluish-green coloration.

RESULTS

Seed Germination:

Direct exposure of white pine seeds to fenitrothion during stratification did not result in significantly less seeds germinating, (Figure 1). The final germination percentage of all three groups was essentially similar. There was an apparent lag between the mean total daily germination percentage of 1000 ppm fenitrothion exposed seeds when compared with those of the control and 10 ppm fenitrothion exposed sets. However, the overall speed of germination when evaluated statistically did not significantly reflect this observation (Table 1).

Table 1

Speed of Germination of White Pine Seeds

Exposed to Fenitrothion During Stratification

Fenitrothion Concentration (ppm)	Total Speed of Germination
0	0.8067 ± 0.1389
10	0.8346 ± 0.1822
1000	0.7237 ± 0.1366

Germination of white pine seeds stratified for 21 days in water then placed for three days in either water, 10 ppm or 1000 ppm fenitrothion demonstrated similar germination characteristics. The fenitrothion concentrations used did not significantly affect the total mean percentage germination (Figure 2). A lag was observed with the 1000 ppm fenitrothion exposed seeds, but was not shown to be statistically

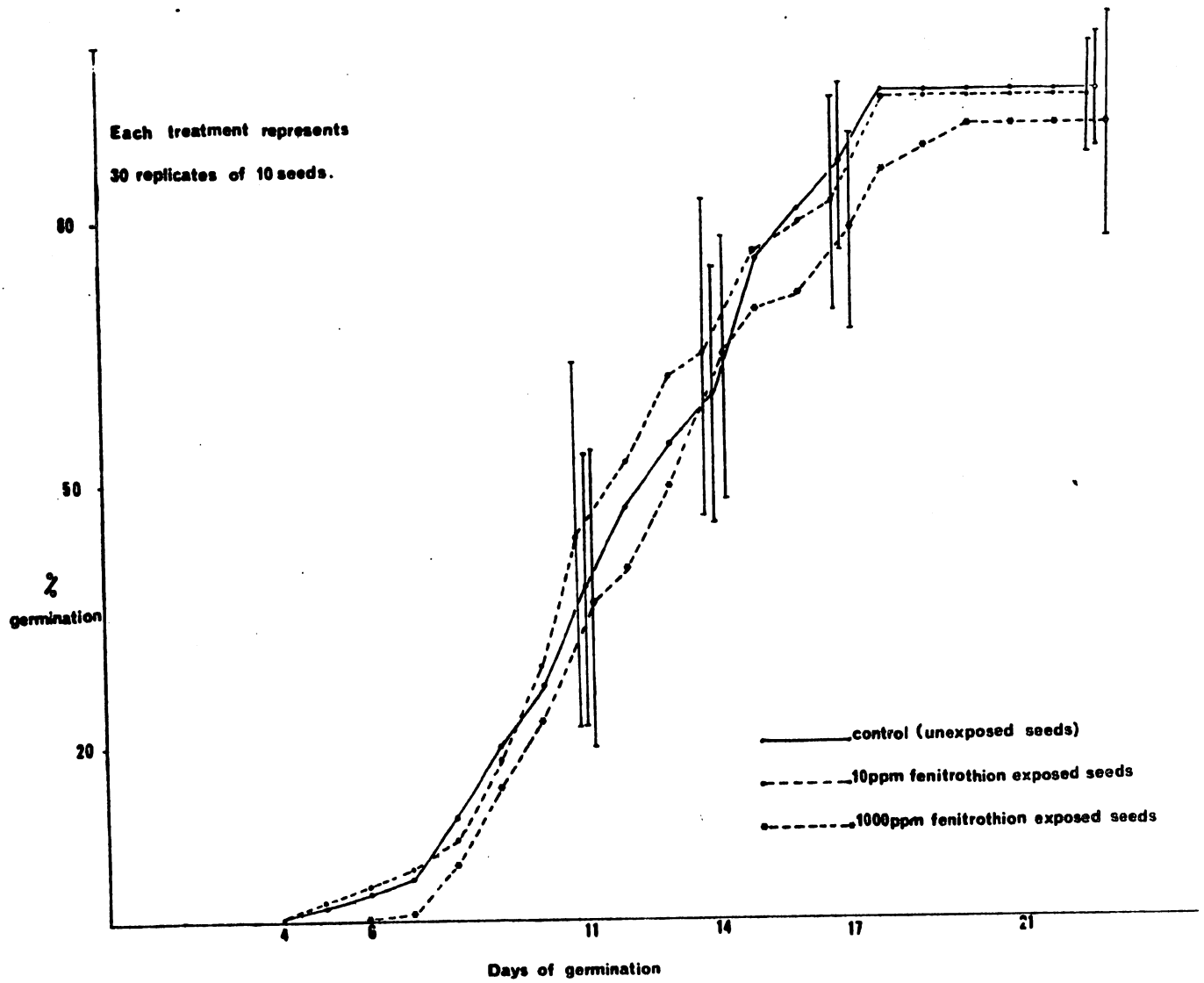


FIGURE 1: Daily germination of white pine seeds following 21 days of stratification and fenitrothion exposure.

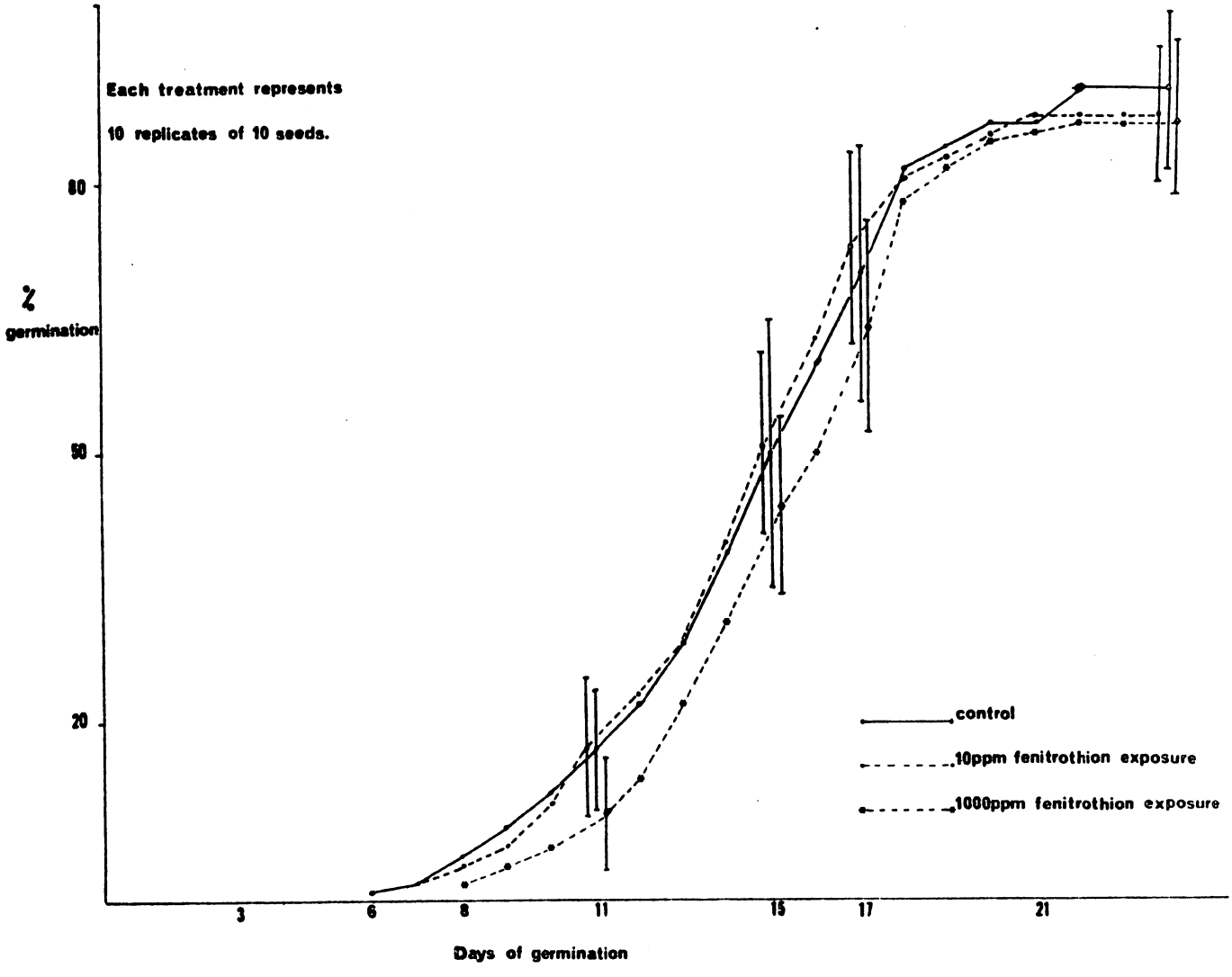


FIGURE 2: White pine germination following three days of fenitrothion exposure in germination conditions.

significant, (Table 2). The final germination percentage of the three groups were essentially similar.

Table II

Mean Total Daily Germination Percentages of White Pine Seeds Exposed to Fenitrothion During the Initial 3 Days of Germination Only.

Fenitrothion Treatment (ppm)	11th Day	15th Day	17th Day	Final %
0	17.0 ± 6.7	50.0 ± 15.6	70.0 ± 14.1	91.0 ± 8.8
10	17.0 ± 8.2	51.0 ± 10.5	74.0 ± 10.7	88.0 ± 7.9
1000	9.0 ± 7.4	44.0 ± 9.7	65.5 ± 12.1	87.0 ± 9.5

Growth Studies:

White pine seedlings derived from seeds exposed to the 10 ppm and 1000 ppm fenitrothion concentrations failed to show any obvious differences from the control seedlings following 5 months of growth.

Water Uptake:

At each time period, white pine seeds exposed to 1000 fenitrothion took up significantly more water than their control counter-parts during stratification (Figure 3). The 10 ppm fenitrothion exposed seeds were found to be intermediate in fresh weight and did not differ significantly from either the control or 10 ppm insecticide exposed treatments. Following 2 days of germination, the fresh weights of all these groups were essentially similar.

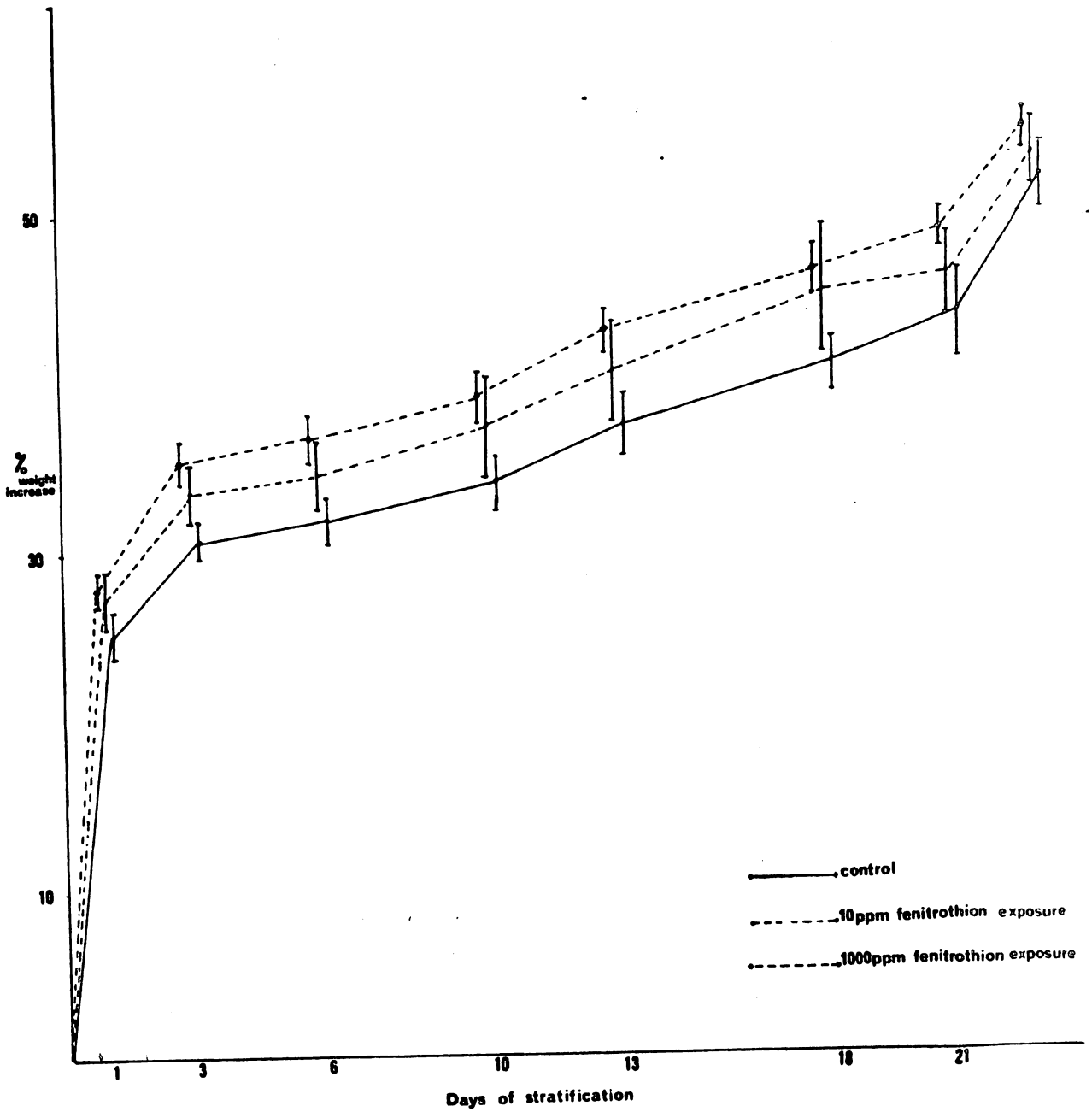


FIGURE 3 Percentage water uptake of stratifying white pine seeds

Oxygen Uptake:

White pine seeds monitored at four different periods (5, 10, 15 and 21 days) during stratification showed no significant deviations between the control and 1000 ppm fenitrothion exposed seeds. In both groups increasing oxygen consumption was evident from day 5 to day 21.

Histochemical Studies on Pine Seeds: Total carbohydrates (insoluble polysaccharides).

The unimbibed white pine embryos had comparatively small amounts of insoluble polysaccharides and starches in the suspensor-root cap region, the cotyledons or shoot apex.

Embryos stratified for 21 days in distilled water, 10 ppm and 1000 ppm fenitrothion showed very intense localization of polysaccharides and starch in the root cap and cotyledon areas (Figs. 4(a) and (b)). However no differences between treatments were apparent.

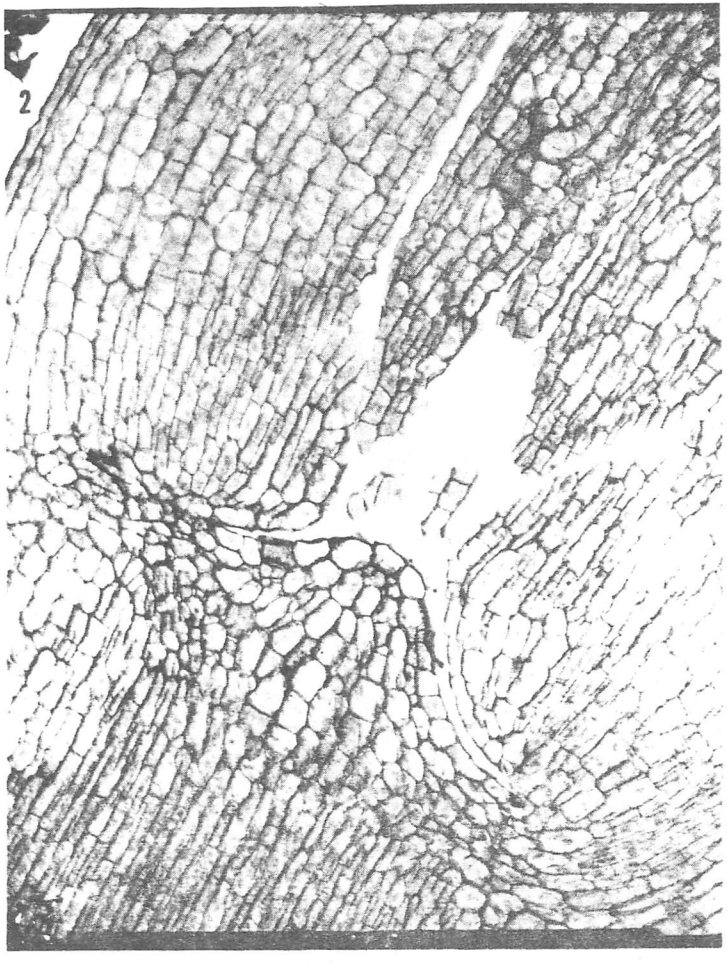
Following 10 days of germination, polysaccharides were localized in the shoot apex and cotyledons although starch could not be detected. There were no observable differences between treatment groups.

DNA:

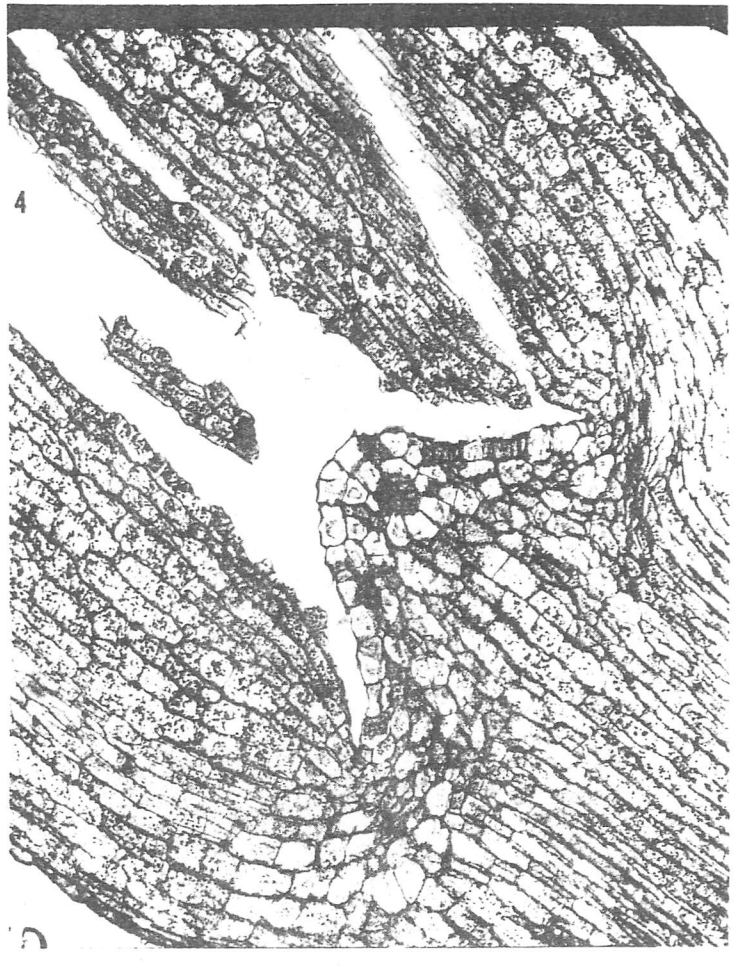
In the unimbibed embryo, large, often irregular shaped nuclei were observed in the apical initials of the shoot which did not stain intensely for DNA in either this region, nor in the root capapical regions.

Following 21 days of stratification, DNA stained intensely in the nuclei of the shoot, and root apical cells (Figs. 5 (a) and (b)). Although the localization was more pronounced at this time, no differences could be determined between treatments.

After 6 days of germination, the nuclei of the root initial cells showed a more rounded shape, and intense localization was observed



(a)



(b)

Fig. 4 Histochemical localization of carbohydrates in shoot apices of white pine - before (a) and after (b) stratification. Note the starch and polysaccharides in (b).

here and in the shoot apical initials. Little change in form and localization from that of 6 day embryos was evident following 8 days of germination. Nuclei in the root apical cells, and cells of the shoot apex, stained intensely for DNA. There was no differences between treatments in DNA localization.

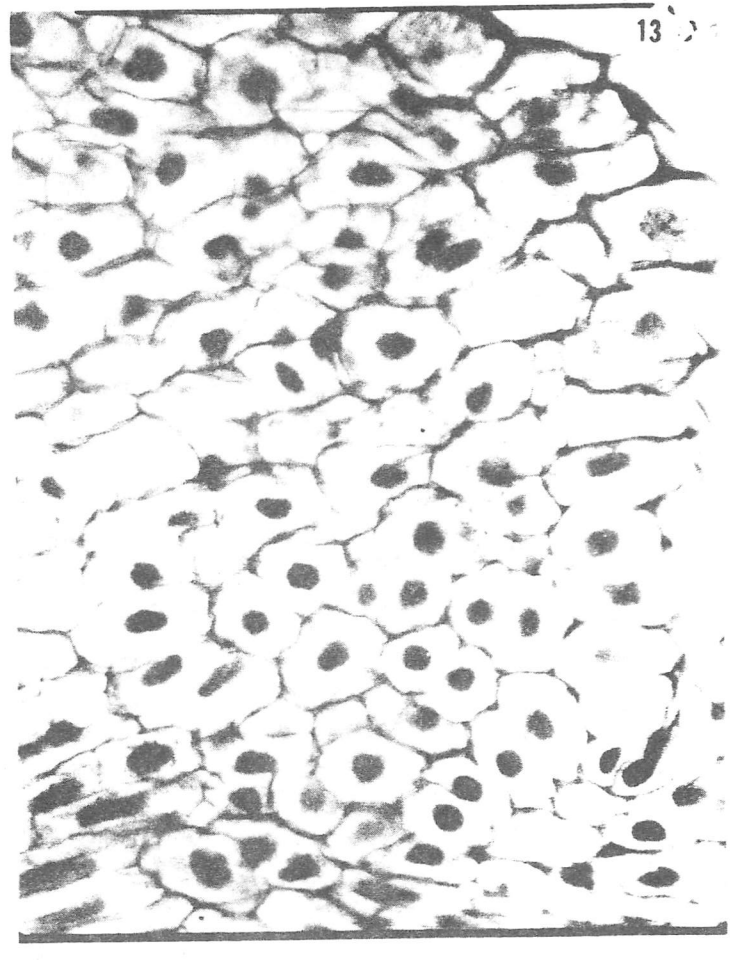
Following 10 days of germination, changes in the nuclei were observed in both the shoot apex and root apex. Increased intensity of DNA localization was observed in all treatments. Control embryos and those exposed to 10 ppm fenitrothion were observed to have more rounded nuclei than those exposed to 1000 ppm fenitrothion. Early stages of mitotic activity could be observed in cells of the root apex in both the control and 10 ppm fenitrothion exposed embryos.

Basic Proteins:

In the unimbibed embryo, cytoplasmic proteins high in basic amino acids were evident in all cells and were localized in discrete bodies in the cells below the shoot apical initials, and root cap region. Nuclear basic proteins (histones) were quite evident.

Following 21 days of stratification, nuclear histone localization paralleled the increased DNA levels in both the root cap and root apical cells and shoot apical cells. Nuclear histone localization did not appear to differ significantly between treatments. Basic proteins did show increased cytoplasmic levels which were most pronounced in the ground meristem tissue beneath the shoot apical initial in embryos exposed to 1000 ppm fenitrothion. These basic proteins were observed to be localized in large globular bodies which occupied most of the cytoplasmic volume of the cell. Cytoplasmic basic proteins were observed to be present in all regions of the embryo, including the shoot apex. Following six days

(a)



(b)

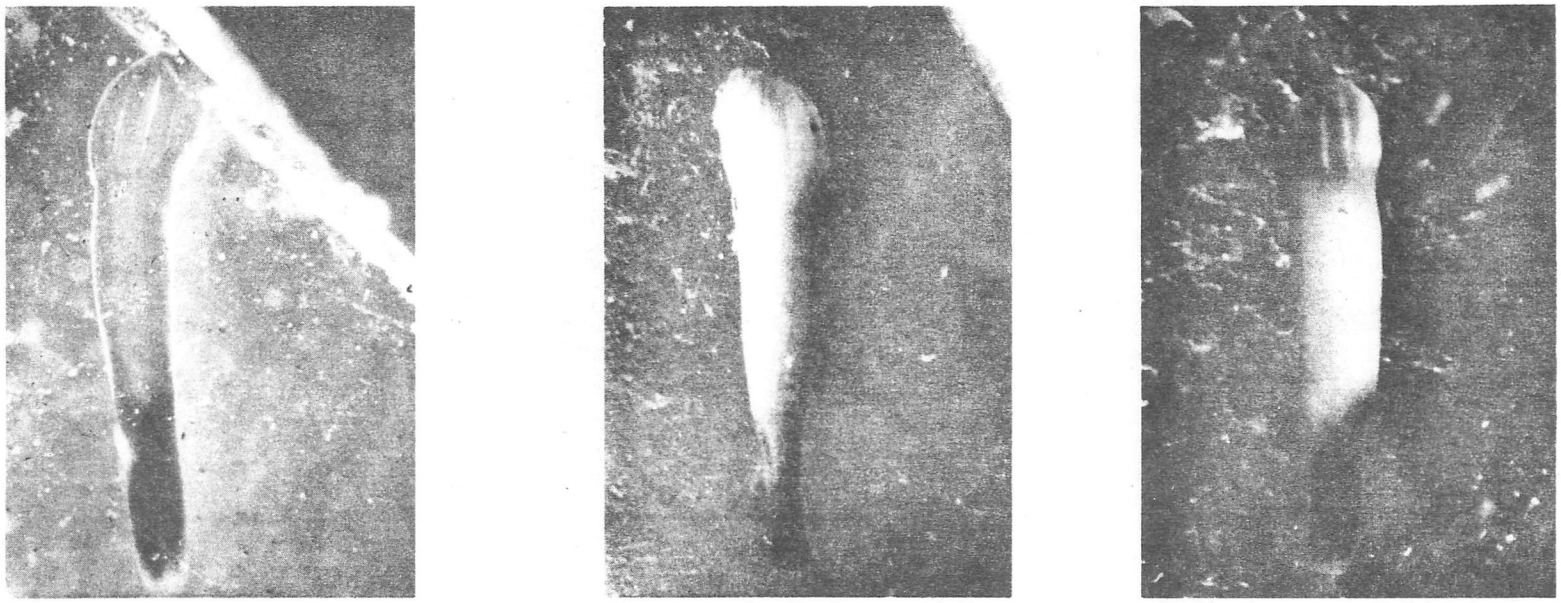
Fig. 5. Histochemical localization of DNA in shoot apices of white pine - before (a), after (b) stratification. Note the dense nuclei rich in DNA in (b).

of germination nuclear histones were found to stain intensely both in the shoot apical cells and cells of the root apex. However, the control and 10 ppm group did not appear to be appreciably different in the distribution or amounts of the nuclear histones. Again, intense localization of cytoplasmic basic proteins was observed in all areas of the embryos exposed to 1000 ppm fenitrothion. Following 8 days of germination nuclear histones stained intensely in all treatment groups in both the shoot apical cells, and root apex. Cytoplasmic basic proteins were localized quite extensively in regions above the root apex, in embryos exposed to 1000 ppm fenitrothion, but were less apparent in these regions in either the control, or 10 ppm fenitrothion exposed groups.

After 10 days of germination nuclear histones did not localize well in either the root apex, or shoot apical cells of the control and 10 ppm fenitrothion exposed embryos. Many nuclei stained a light green-yellow colour whereas nucelar histones of the 1000 ppm fenitrothion exposed embryos showed intense blue staining in the shoot and root apices. The distinct cytoplasmic basic proteins observed at earlier periods were not now apparent. However, the cytoplasm did stain lightly in the 1000 ppm exposed embryos (see Figs 6, a, b and c).

Esterases:

White pine embryos were stratified for 14 days and stained to localize esterases. A deep blue colour indicated the site of esterase activity. The control embryos stained heavily throughout, whereas those exposed to a 10 ppm fenitrothion concentration stained heavily but often in a more patchy manner in the hypocotyl region. Embryos exposed to a 1000 ppm stained intensely in the root suspensor region but less extensively in the hypocotyl and cotyledons.



(a) (b) (c)

Fig. 6. Distribution of esterases in white pine embryo following exposure to fenitrothion.
(a) control (b) 10 ppm (c) 1000 ppm.

Esterase localization following 21 days of stratification was intense in the root-suspensor region but less so in those that had been stratified for 14 days. The embryos exposed to 10 ppm fenitrothion stained less heavily in the cotyledons, than those which were unexposed to fenitrothion. Embryos exposed to 1000 ppm fenitrothion showed a decreased esterase stain in the hypocotyl.

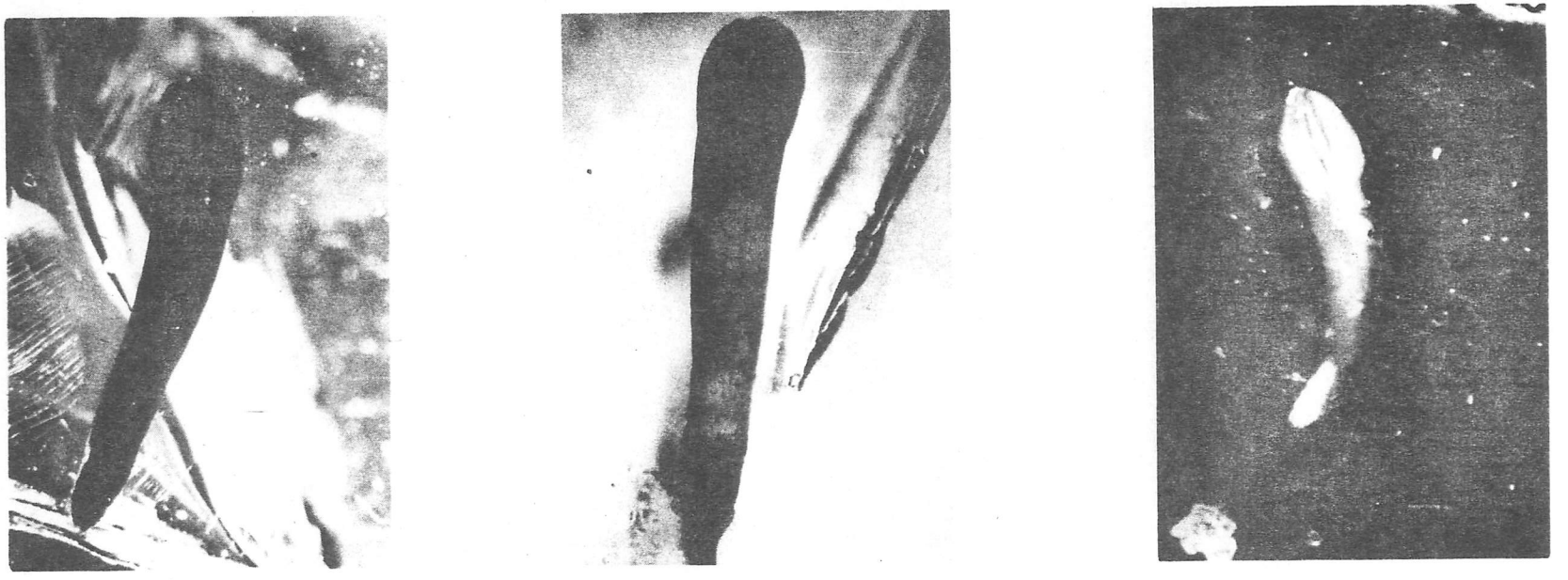
Succinic dehydrogenases:

White pine embryos stratified for 21 days and stained for succinic dehydrogenases showed a deep staining pattern throughout, whereas embryos exposed to a 10 ppm fenitrothion concentration showed staining only in the root suspensor regions and cotyledons. Embryos exposed to 1000 ppm fenitrothion did not demonstrate the presence of the enzyme at all (see Figs 7, a, b and c).

DISCUSSION

Five month old white pine seedlings derived from seeds exposed to fenitrothion during stratification did not show any noticeable long term morphological effects. Although seeds exposed to 1000 ppm fenitrothion, either during stratification or in the initial period of germination, did result in a consistent indication of suppressed germination percentages, this was not significant at the 95% level.

The metabolic studies did indicate that changes were occuring on the molecular level. However, it was not possible to determine whether these observed alterations could be directly linked to fenitrothion exposure and/or its toxic breakdown products, or rather symptoms



(a) (b) (c)

Fig. 7. Distribution of succinic dehydrogenases in white pine embryo following treatment with fenitrothion. (a) control, (b) 10 ppm, (c) heat killed embryo. Light colour indicates absence and dark colour indicates presence of the enzyme.

of specific pesticide-metabolic interactions. For example, it is apparent that normal respiration is occurring during the stratification of white pine seeds exposed to the insecticide, however localization of succinic dehydrogenase was less intense. Molnar (1972) reported a lack of succinic dehydrogenase localization in tissues staining positively for cytochrome oxidase and obviously respiring. He suggested the presence of strong inhibitors in the cells or high levels of peroxidase which would effectively mask the enzyme activity. One might suspect a similar situation in white pine embryos exposed to high concentrations of fenitrothion, perhaps as a result of increased peroxidae levels due to the oxidation of fenitrothion to fenitro-oxon (Hallett, Weinberger, Greenhalgh and Prasad 1974).

The relationship of peroxidae in this situation is not clear but certainly worth investigation. Decreased localization of esterases suggests specific interaction of the insecticide (a cholinesterase inhibitor) with the enzymes. Cholinesterases have been isolated from plant tissues, however their exact function in these cells has not been elucidated (Riov and Jaffe, 1973). Cholinesterases have been shown to be inhibited by plant growth retardants causing a buildup of endogenous growth retardants such as acetylcholine (Riov, 1973; Holm and Miller, 1972) which effects gibberellic acid synthesis (Riov and Jaffe 1973). The possibility that acetylcholine is involved in membrane permeability and ion transport in some plants (Bennett-Clark, 1956) may also account for some of the effects of fenitrothion behaving as a cholinesterase inhibitor at high concentrations and therefore disrupting normal ion flux. Further work along these lines should clarify this problem.

Evidence of metabolic change in white pine was indicated by the concentration of cytoplasmic basic proteins as evidenced by the intensity of staining of the cells of the embryos exposed to 1000 ppm fenitrothion. Cytoplasmic basic proteins have been generally found in germinating conifer embryos (Riding and Gifford, 1973) which have had quite high arginine levels and serve presumably as storage proteins (Durzan, et al 1971). The high levels of basic proteins observed during the germination of embryos exposed to 1000 ppm fenitrothion thus suggest a lower utilization rate of these substances than in the unexposed or 10 ppm exposed embryos.

SUMMARY AND CONCLUSION

Seeds of white pine were stratified in water or a water-fenitrothion emulsion. Two concentrations of fenitrothion were used, namely 10 ppm and 1000 ppm. An increase in water uptake and basic proteins (histones) were only observed in those seeds exposed to 1000 ppm fenitrothion. However, some decreased enzymatic activity (succinic dehydrogenases and esterases) as evidenced by localization techniques was observed primarily in the hypocotyl region of the embryo following exposure to both concentrations of fenitrothion. There was no apparent effect on total insoluble polysaccharides or on DNA metabolism up to 10 days of germination. Germination percentages and rates did not differ significantly among the control or treated groups. At the end of five months, white pine seedlings exposed to the insecticide showed no noticeable morphogenetic nor developmental growth defects.

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