FATE OF FENITROTHION IN FOREST TREES

III. Development of a Histoautoradiographic Technique for Investigating Foliar Penetration in Balsam Fir and White Spruce

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RÉSUMÉ

On a fait une étude histo-autoradiographique afin de localiser les résidus de fénitrothion-C^{14} dans le sapin baumier et l'épinette blanche. A cause de sa solubilité dans nombre de solvants couramment utilisés en histologie, les méthodes ordinaires d'inclusion des pièces dans les paraffines et dans les polyéthylène glycols n'ont pas donné de résultats satisfaisants pour ce composé. Par contre, la méthode de coupe à température optimale à l'aide d'un microtome type cryostat s'est révélée la plus commode, tandis que le meilleur agent de protection contre le gel des tissus de conifères a été une solution de glycérol à 10%. Le prétraitement des tissus au moyen de cette solution a donné des coupes reproductibles et de bonne qualité.

La radioactivité (à l'origine de la formation des grains d'argent) persiste dans la cuticule, l'épiderme et l'hypoderme des aiguilles des deux espèces pendant 21 jours, et il se produit un transport vers les tissus internes (mésoderme et système vasculaire). On a aussi trouvé des grains d'argent dans les vaisseaux du bois des tiges de sapin baumier et d'épinette, ainsi que dans les régions épidermiques et hypodermiques des tissus fasciculés.

INTRODUCTION

The insecticide, fenitrothion (0,0-dimethyl 0-(4 nitro-m-tolyl phosphorothioate) has become the major replacement of DDT for protecting the Canadian forests against the ravages of the spruce budworm (Fettes 1968). According to Yule and Duffy (1972), although fenitrothion is largely degradable in the forest environment, it does persist in coniferous foliage for at least one year following aerial applications @ 4-6 oz/acre. They did not elaborate on the mechanisms of persistence but thought that fenitrothion probably gets bound in the cuticle layer. Similarly, Hallett, Weinberger, and Prasad (1973) observed considerable accumulation of fenitrothion and its metabolites in the embryo, perisperm and seedcoat of treated seeds of eastern white pine (Pinus strobus L.). That fenitrothion may have a systemic action in controlling the spruce budworm during early spring spraying was first suggested by Randall (1970). He speculated that after the aerial application, fenitrothion deposits on the spruce buds, penetrates through the sheath and causes acute toxicity and mortality to the emerging larvae of the spruce budworm.

This report outlines the development of an autoradiographic technique for tracing the penetration and movement of ring labelled fenitrothion - C^{14} in white spruce and balsam fir seedlings. A further report (Prasad, Sundaram & Yule (1974)) on the practical results of this study together with a quantitative aspect of the chemical and radiochemical metabolism of fenitrothion is in preparation.

MATERIALS AND METHODS

Culture of Trees

Three year old seedlings of white spruce (Picea glauca L.) Mill and balsam fir (Abies balsamea (Moench) (Voss) were obtained from the nursery (Ontario Ministry of Natural Resources, Kemptville) in August, 1973 and were held outdoors in potted soil until December (1973) before being transferred to a greenhouse. It was found that this period of exposure to chill was adequate to break dormancy, as the vegetative buds started to flush immediately after the trees were moved into the greenhouse set at 16 hr. photoperiod, $72^{o} \pm 2^{o}$ and 50 ± 20% relative humidity. Illumination was achieved with fluorescent daylight tubes at about 2000 foot candles. The pots were regularly irrigated with a half strength Hoagland solution and under these conditions, the new buds broke dormancy and the needle growth was profuse and excellent.

Insecticide Treatment

Fenitrothion premium grade was obtained from the Sumitomo Chemical Company, Japan. C^{14} labelled material was synthesized by Dr. D.J. Duffy of the Prince Edward Island University with the label introduced into the ring (cresol) position with a specific activity of approx. 10 mc/mM. Before use, the chemical and radiochemical purities of both samples were checked and analysed. To represent the field concentrations an emulsion of cold fenitrothion, (fenitrothion-10% v/v, Aerotex 3470-1% v/v, Atlox 3409-1% v/v and distilled H_2O 88%) was prepared to which 10 µc of the labelled material was added. Four sets of branches

per tree were dipped in this mixture to ensure a uniform coverage. To avoid contamination due to run-off & drainage, a serum cap was snugly fitted to the lower branch. There were two replicates of each treatment and only newly formed needles were used for treatment. Care was taken not to contaminate the soil & other parts of the tree. Therefore, a plastic sheet was used to cover the soil in the pot. Samples of foliage were taken periodically (1, 4, 16 and 21 days) for chemical determination of residues on and within the tissues. Balsam fir foliage was found to be somewhat easier to dip than that of spruce. An appropriate control tree was always included in each test. All experiments were conducted in the greenhouse under the above mentioned conditions.

Method of Sectioning

Various embedding and sectioning techniques were tested in an attempt to obtain the most suitable method: the primary aim being to prepare tissues in such a way that would preserve cellular detail and organization without producing artifacts. In accordance, all samples were first fixed in the standard formalin acetic acid (F.A.A.) for at least 48 hours. Three methods of embedding (paraffin, carbowax and optimal cutting temperature, O.C.T. Compound) were tried, the details of which are described below:

(i) Paraffin Embedding: is the standard method recommended for various plant materials (Jensen 1962); the tissue was first washed in brief changes of 50% ethanol and cut into 3-4 mm segments, and then passed through a tertiary butyl alcohol (TBA) series as shown in Table 1.

TABLE I

Procedure for Dehydration of Balsam fir & Spruce Needles in Tertiary Butyl Alcohol (TBA) Series

Concentration of Alcohol (%)	Preparation	Time of Immersion
50	50 ml H_2O, 40 ml 95% ethanol, 10 ml TBA	1 hour
70	30 ml H_2O, 50 ml 95% ethanol, 20 ml TBA	1 hour
85	15 ml H_2O, 50 ml 95% ethanol, 35 ml TBA	1
95	45 ml 95% ethanol, 55 ml TBA	1
100	25 ml 95% ethanol, 75 ml TBA	1
TBA-I	100%	1
TBA-II	100%	3
TBA-III	100%	8

After the necessary dehydration, the tissue was placed in sufficient TBA contained in a vial half filled with solid Tissuemat paraffin (m.p. 56.5, Fischer Scientific Co.) and this vial was then placed in an oven at 60° C. Paraffin was decanted and renewed 4 hours after melting and the vial was replaced in the oven for a further 4 hours. The paraffin was again decanted and renewed for 8 hours after which the infiltrated tissues were embedded in fresh paraffin and sectioned at 7 μ in a rotary microtome (Leitz model). See Fig. 1 (a) and (b).

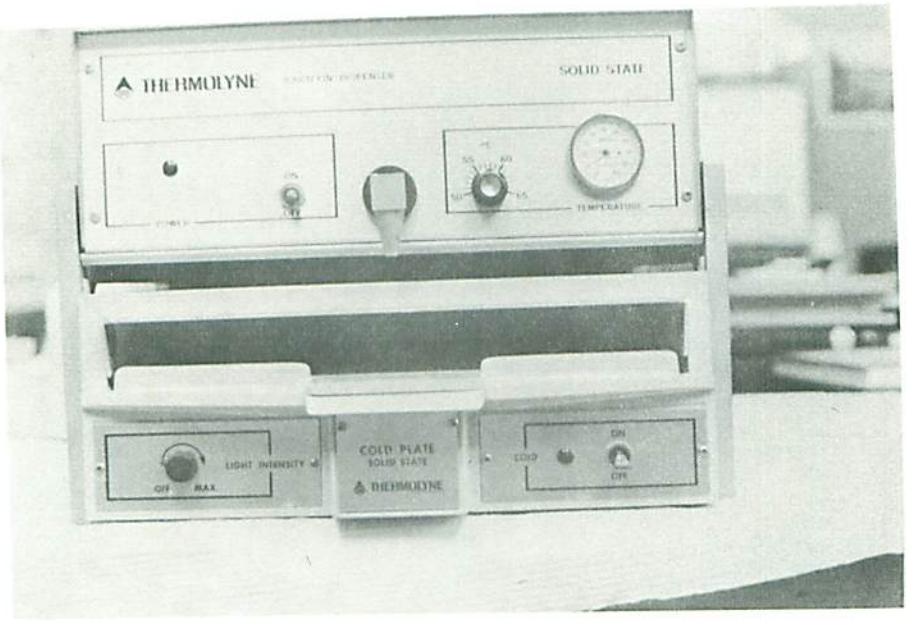


Fig. 1(a) A paraffin dispenser used for embedding balsam fir

Fig. 1(b) Paraffin sectioning with a Rotary Microtome. The paraffin ribbon containing the sections were lifted up on a paper and then gently placed on the slides.

Sections were mounted on glass slides smeared with Tissue-Tek adhesive (Fischer Scientific Co.) and deparaffinized by a 5-minute immersion in xylene followed by a further 5 minute immersion in a mixture of xylene and ethanol (1:1 v/v). Subsequently, all sections were rehydrated by passing the slides through an ethanol and water series (50, 75, 85 and 100% water), 2 minutes in each solution. The rehydrated sections were finally subjected for autoradiography.

(ii) Carbowax Embedding: Because fenitrothion is readily soluble in histological solvents (TBA, xylene) considerable leaching and loss of radioactivity took place from the paraffin embedding technique. Therefore, the carbowax (polyethylene glycol) embedding procedure was resorted to as no dehydration of sections was necessary. Briefly, the needles were washed in several changes of 50% ethanol and cut into 3-4 mm segments. Two brands of carbowax, mol. wt, 1500 and 4000 were mixed in a ratio of 2:10 and heated to 175^{o} C to expel any moisture present and then stored at 56^{o} C until used. The needles were finally immersed in the molten carbowax mixture and left at 56^{o} C for 8 hours for infiltration after which they were re-embedded in fresh molten carbowax. Using a rotary microtome, sections of various thickness were cut from the blocks until a suitable section was achieved.

(iii) Optimal Cutting Temperature (O.C.T.) Compound Embedding: The carbowax method did not yield clean-cut sections: rather the tissues were fragmented and anatomical identity of sections was distorted, therefore, in order to minimize artifacts and disruptions, frozen sectioning was attempted. For this, balsam fir and spruce needle and stem were severed into 3-4 mm segments and soaked overnight in a 10% glycerol solution. Then they were aligned and oriented properly in a liquid O.C.T. compound (Fischer Scientific Co.) which, in turn, was frozen in the freezing bar of an Ames II cryostat. (See Fig. 2 and the Appendix). Sections (10 μm) were cut at -23° C and mounted simply by touching a glass slide to the edge of the ribbon. The O.C.T. compound was then removed by washing the slides in running water for 1 hour. This is necessary for proper adhesion of the radio-autographic emulsion.

Autoradiography

Standard methods of historadiography were adopted from the work of Jensen (1962), Humason (1962) and Fisher & Warner (1971). The Con-Rad/Joftes fluid emulsion system (1955) was employed with appropriate directions being provided by the manufacturer. The slides bearing tissue sections were dipped in Kodak nuclear track emulsion (NTB2) that had been warmed gradually to 40° C. The slides were drained vertically for 10 seconds (Morris 1968) and then left in the exposure chamber for 14 days (see Fig. 3). After exposure, they were developed, fixed, cleared and made permanent.

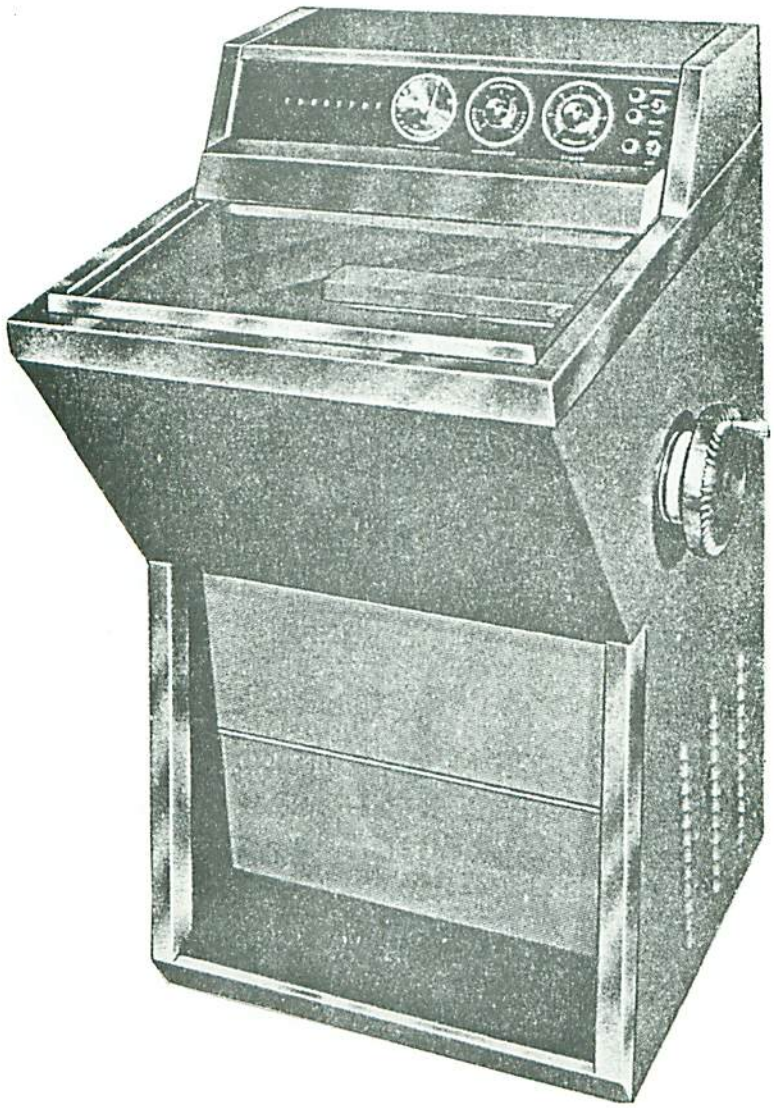


Fig. 2. A cryostat for cutting frozen sections.

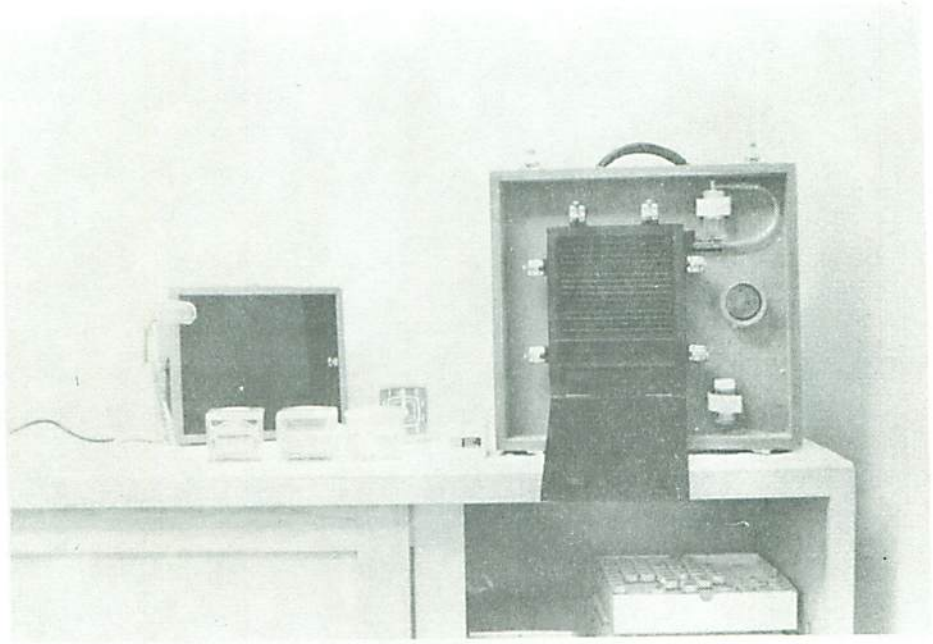


Fig. 3. The Con-Rad/Joftes Model adapted for autoradiography of coniferous tissue.

RESULTS AND DISCUSSION

(i) Paraffin Embedding

Sectioning of the paraffin embedded tissues of balsam fir and spruce yielded excellent microscopic specimens; the anatomical features were intact and clearly defined. (See Fig. 4(a), (b)). However, the developed autoradiograms exhibited little evidence of the presence of radioactivity (silver grains) suggesting that much of the C^{14} labelled compound(s) had been leached from the tissues during preparation. Indeed, scintillation counting of the solutions used for dehydration and deparaffinisation (TBA, xylene) demonstrated presence of considerable activity. For this reason, this method was discarded.

(ii) Carbowax Embedding

Even after repeated attempts, no firm and good sections were obtained by this method. It seemed as if poor infiltration of the carbowax into the tissues was responsible for this. Various attempts were made to alter the duration of infiltration and microtome settings but none was successful. Since coniferous tissues are heavily coated and impregnated with aromatic and lipid compounds, it seemed penetration of water soluble carbowax was difficult and hence no good sections were obtained by this procedure.

(iii) O.C.T. Compound Embedding

This method provided satisfactory microscopic specimens (see Figs. 5 & 6) which displayed strong and localized radio-activity in the cells. There were

large concentrations of silver grains and the results consistently revealed the presence of C^{14} molecules in the epidermis and hypodermis of treated balsam fir and spruce needle (See Fig. 7 & 8). At times, some translocation of the compound(s) through the xylem vessels of the needles was also observed. This is clearly seen in Figs. 9, 10 and 11. Whether radioactivity could cross the vascular bundles of the needle and translocate to the stem of the seedlings was also investigated. Cross sections of treated stems of spruce exhibited strong activity in the outer epidermis followed by localization into the xylem cavity of the trunk (see Figs. 7-11.) On the other hand, specimens of cross sections prepared from the untreated branches did not demonstrate any silver grains. Therefore, the chances of artifacts resulting from the fixation and sectioning procedures seemed remote and the localized granules must be the authentic fenitrothion or its products. During cryostat microtomy, it is essential that there are no water residues associated with the sections otherwise freezing and formation of ice crystals takes place and the resultant sections tend to be distorted and full of artifacts. Antifreeze treatments with dimethylsulfoxide (DMSO) and glycerol have produced striking effects on the pattern of ice crystal formation. Pease (1966 and 67) has shown that antifreezes or rather cryoprotective agents (glycerol and DMSO) are very effective dehydrating agents; as their concentration is increased, water in the tissue becomes immobilized. Thus when water is present in the tissue it no longer can act as a solvent. Thus during freezing, an antifreeze molecule can substitute for a water molecule in the tetrahedral water lattices. The net result is water loss by diffusion and a change in the nature of crystal formation,

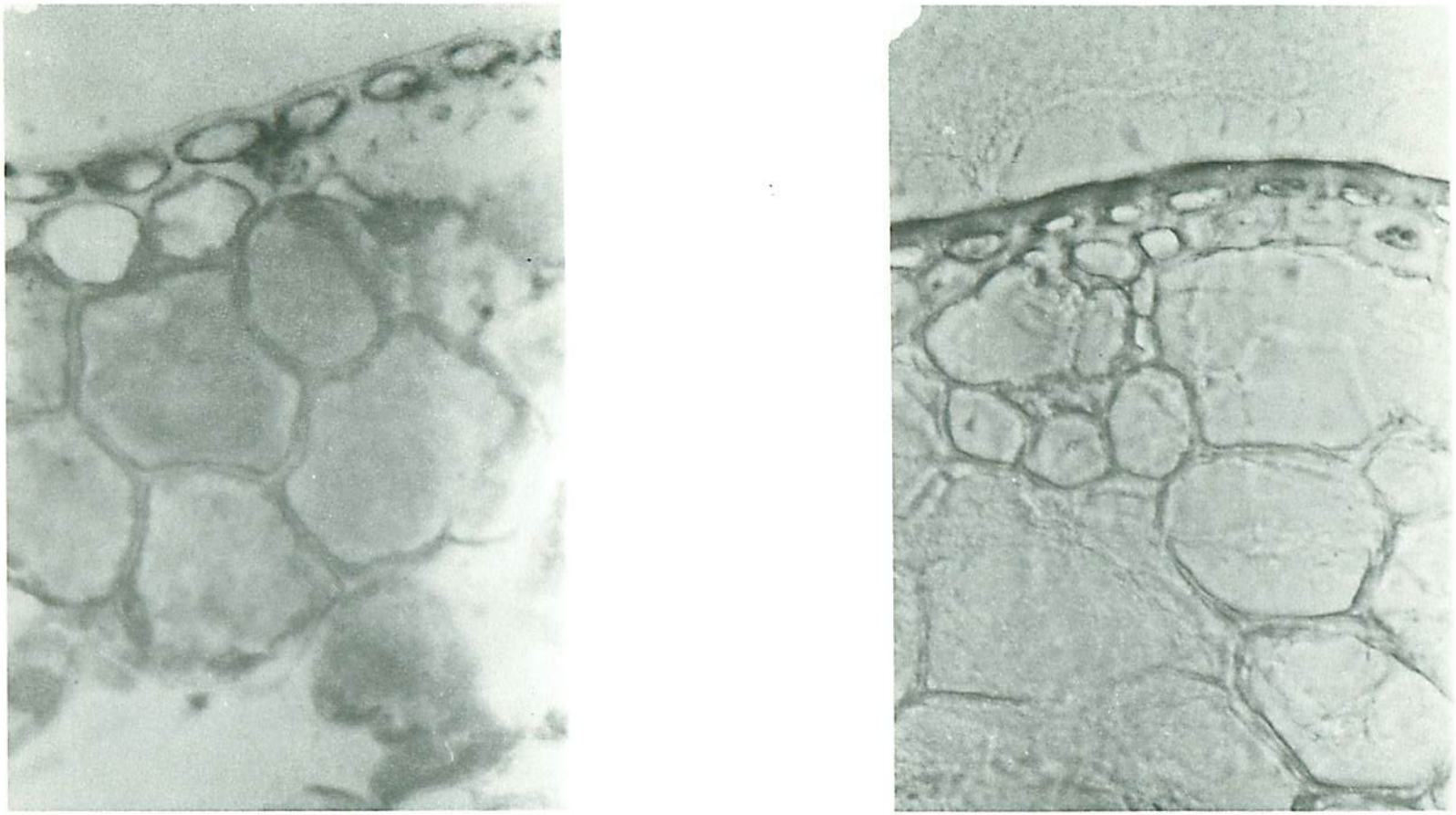
thus explaining the effects of the antifreeze pretreatments during freeze sectioning. As a result of the glycerol (10%) treatment, both balsam fir and spruce needles (Figs. 5(a) and (b)) and stems (Fig. 6(a) and (b)) yielded excellent sections.

Both species (balsam fir and spruce) show the same general trend in accumulation of the label, that is most of the radioactivity seems to be concentrated in the cuticle, epidermis and hypodermis of the needles (Fig. 7 & 8, (a) (b) and (c)). In the case of spruce needles (Figs. 7(a) (b) and (c)), there are more silver granules in the mesoderm (pallisade cells) area at 4 day than at 21 day treatment, probably due to rapid diffusion from the cuticle (Martin and Juniper 1971). Subsequently with the passage of time the activity is transported to the vascular bundles. But most of the grains are localized in the epidermis and hypodermis and thus these findings are similar to penetration of 2,4 D in cuticles of agricultural crops (Norris 1974). Some accumulation takes place inside the cells, some between the cells and some on the cell walls. In balsam fir needle (Fig. 9(a), (b) and (c)) considerable migration of activity (silver granules) took place across the mesoderm and the vascular systems. The xylem-phloem region (Fig. 9(c)) exhibited many silver grains. Similarly xylem regions of both spruce and balsam fir stems contained a very high number of silver grains (Fig.10(a), (b) and (c)). This suggests that fenitrothion or a product thereof (Prasad, Sundaram & Yule 1974) can penetrate and move across the barrier of cuticle, epidermis and hypodermis and finally culminate in the vascular channels of the trees. If this is

the case, then some degree of systemic action of fenitrothion is possible and Randall's (1970) field hypothesis might be held tenable.

Another interesting feature that emerges from these findings is the accumulation of radioactivity in the epidermal and hypodermal region of the fascicle of spruce (Fig. 11(a), (b) and (c)). As illustrated in the figures, there are numerous silver grains in the hypodermal region but this may have been a result of run-off from the point of application at the needles. While the needles were being dipped it is likely some activity leaked into the fascicular tissue. But whatever the case, the evidence (Fig. 11(a), (b) and (c)) clearly demonstrates that fenitrothion preferentially accumulated into epidermis and hypodermis whether it is applied at the sites of needles or the fascicle.

In conclusion these preliminary experiments suggest that the O.C.T. compound (Tissue-Tek) embedding together with cryostatic microtomy, is the best technique for localization of fenitrothion residues in balsam fir and white spruce. More work is needed to confirm the identity of the silver granules together with refinements in cryostatic techniques for improving resolution and minimizing displacement of molecules during autoradiography. For example, Appleton's (1964) procedure might provide a better method of tackling such problems.



(a) (b)

Fig. 4. Transverse section (x400) through spruce needle after being fed (a) with and (b) without fenitrothion-C^{14}. Paraffin embedding. Note the complete absence of silver granules from the cuticle, epidermis, hypodermis and mesophyll cells due to extensive leaching by solvents.

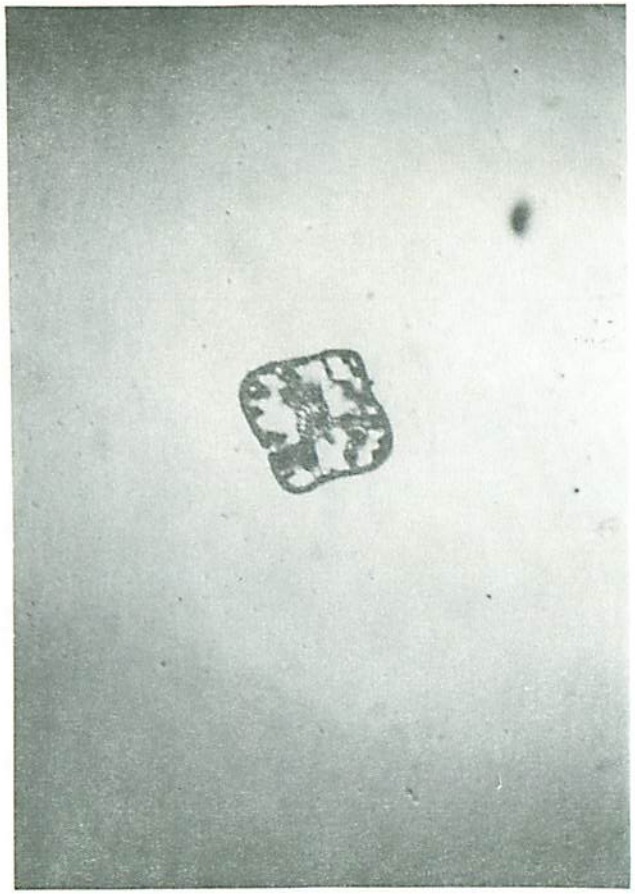


Fig. 5(a) Transverse section through a spruce needle (x 40) showing disrupted cells without any cryoprotective (10% glycerol) pretreatment.

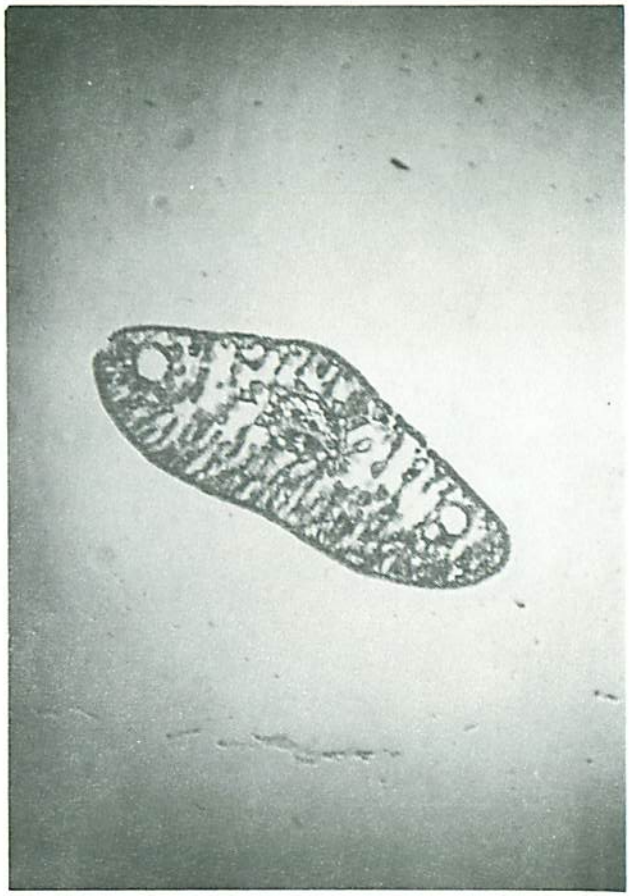


Fig. 5(b) Cryostat transverse section through a balsam fir needle (x 40) after pretreatment with 10% glycerol. Note the integrity of the anatomical structures and distally placed resin ducts.

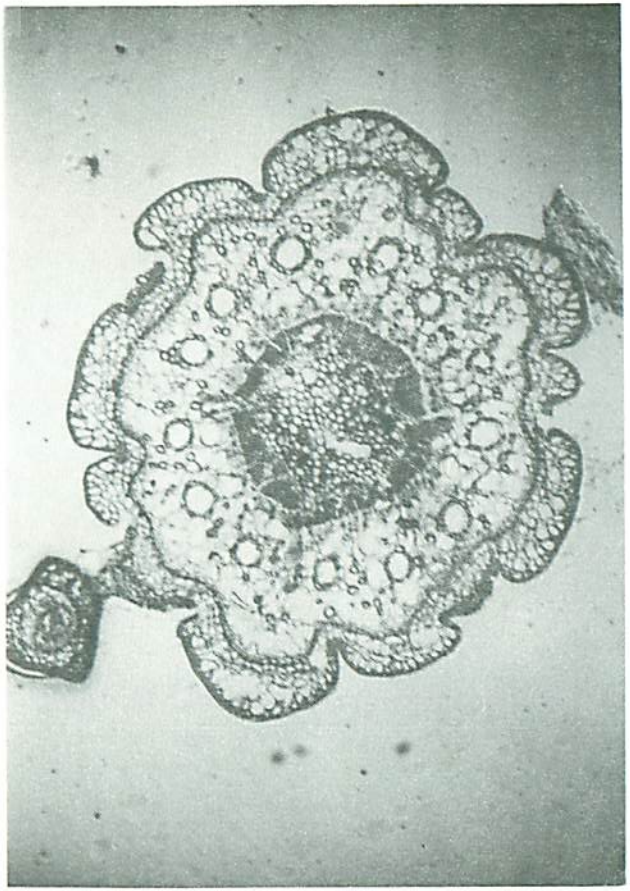


Fig. 6(a) A transverse section through the rachis of spruce. Note the arrangement of resin ducts around the secondary vascular tissues. The outermost layer is the fascicular tissue. (x 40).

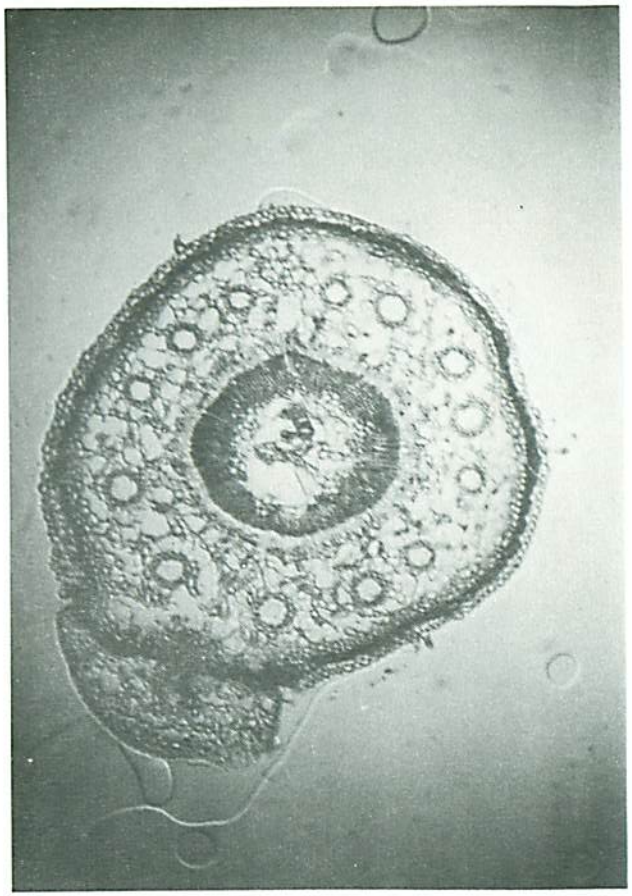
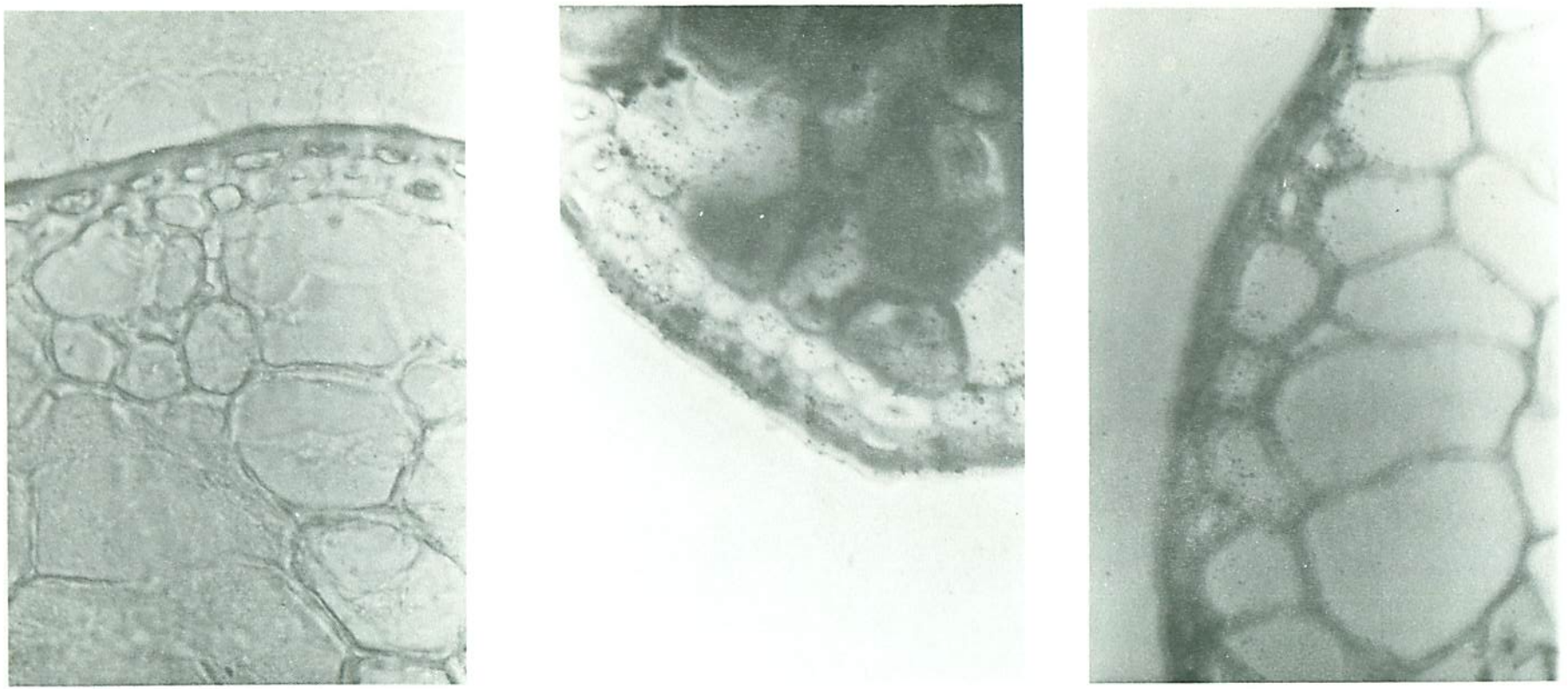
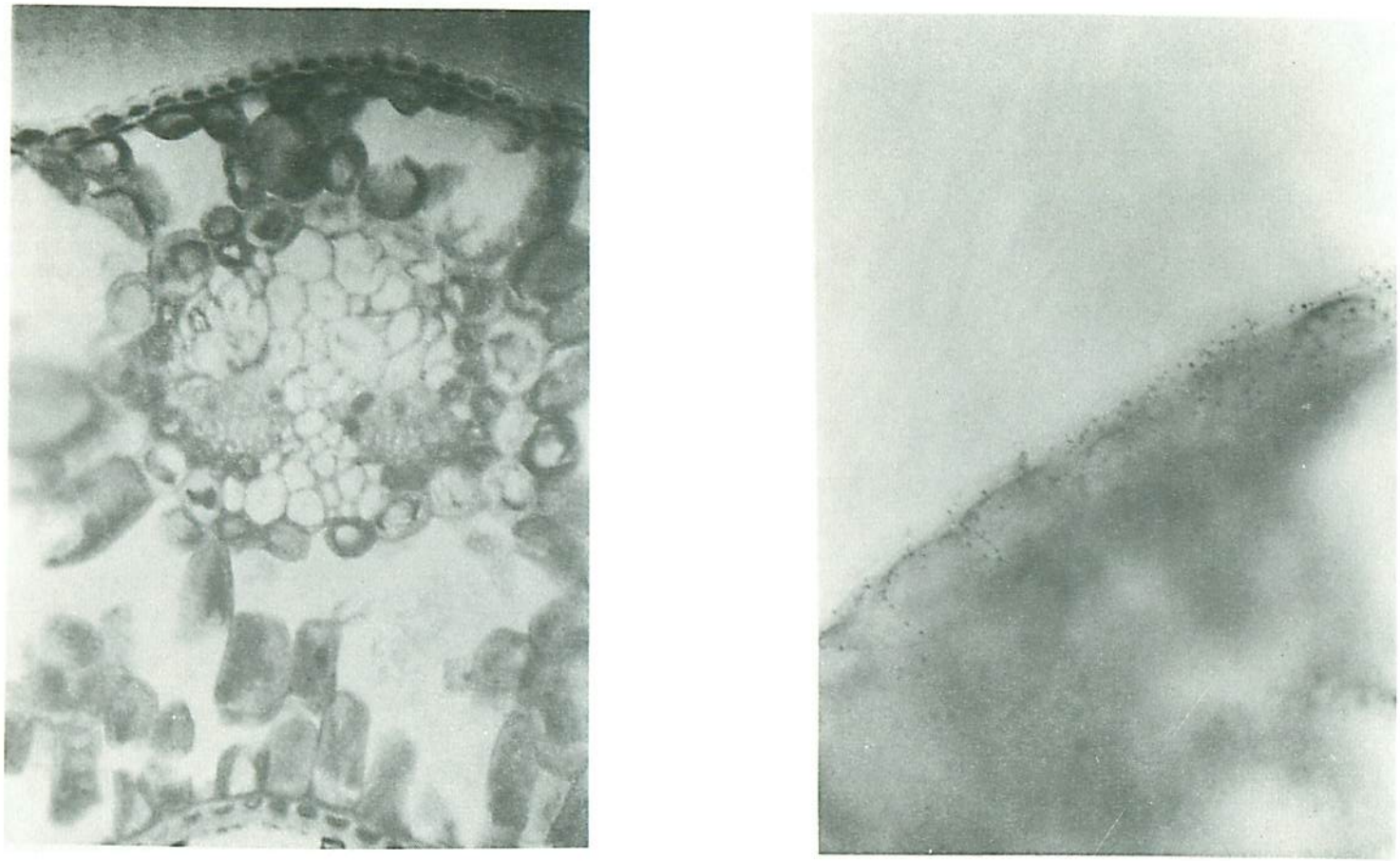


Fig. 6(b) A cryostat transverse section through the rachis of the balsam fir. Note the integrity of anatomical structures and the symmetrical arrangement of the resin ducts (x 40).



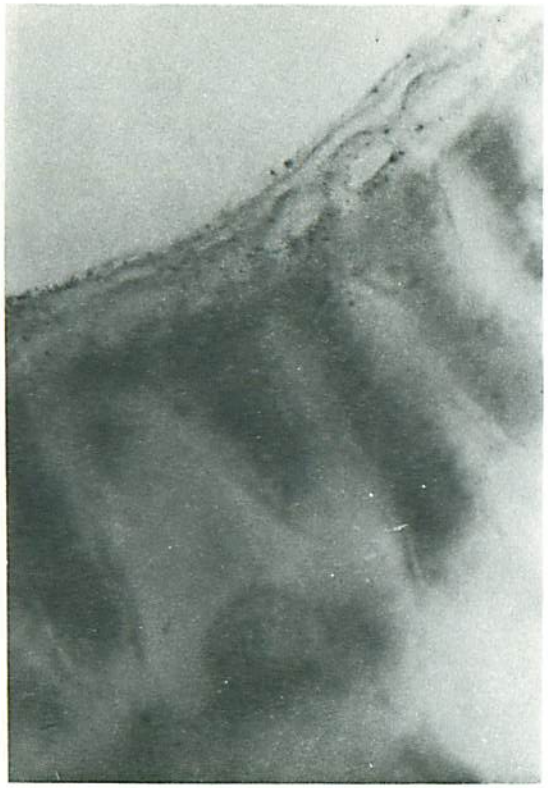
(a) (b) (c)

Fig. 7. Histoautographic localization of fenitrothion - C^{14} in spruce needles: (a) control, (b) 4 day (c) 21 days sample treated with radioactive fenitrothion. Note the complete absence of silver grains in control needles and profuse labelling of cuticle and hypodermis and meosophyll with silver grains in (b) and (c). (x 400).

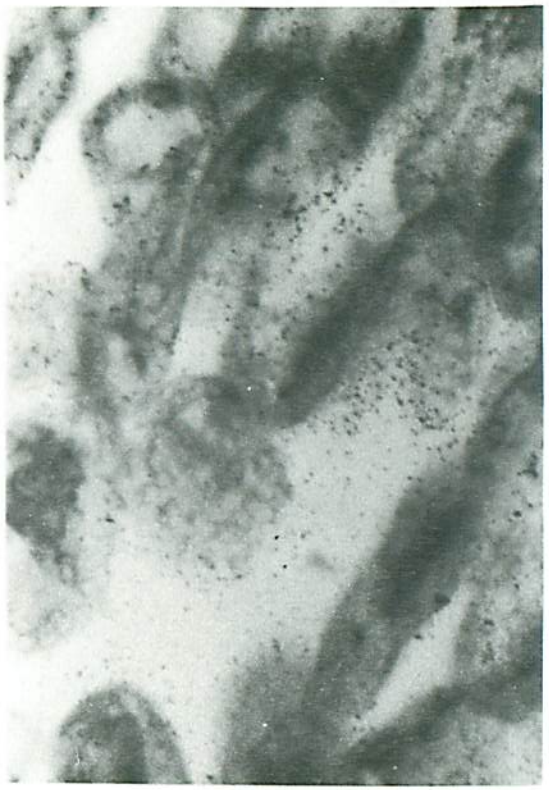


(a) x 100 (b) x 400

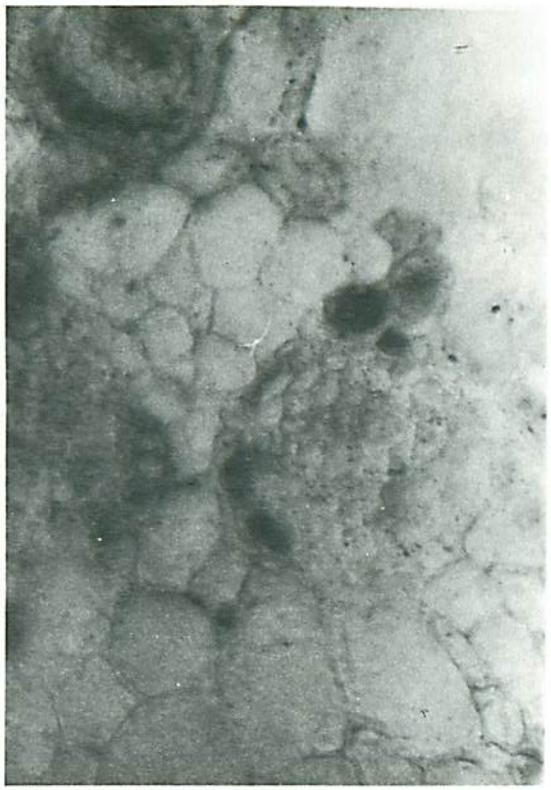
Fig. 8 Histoautographic localization of fenitrothion - C^{14} in balsam fir needles: (a) control (b) 1 day. Note the absence of activity in the mesoderm.



(a) x 400

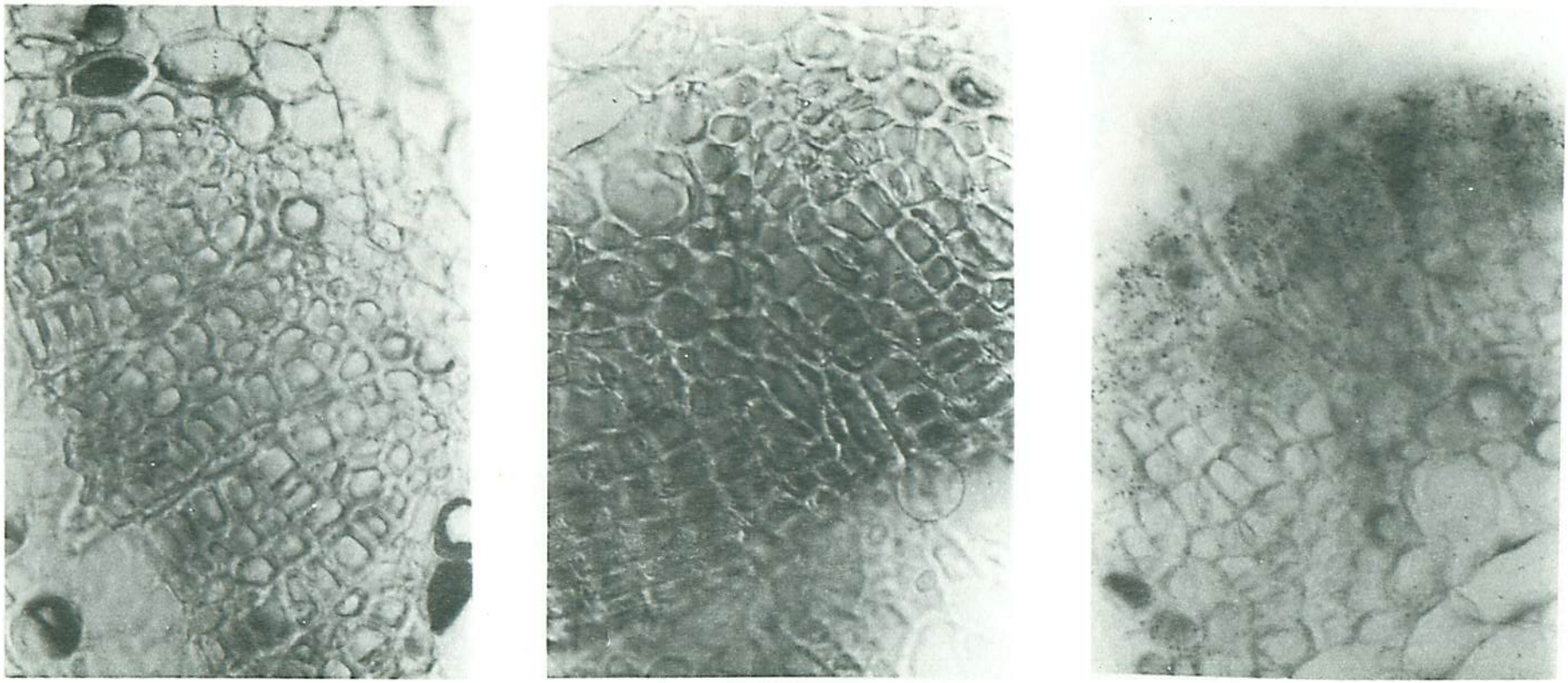


(b) x 400



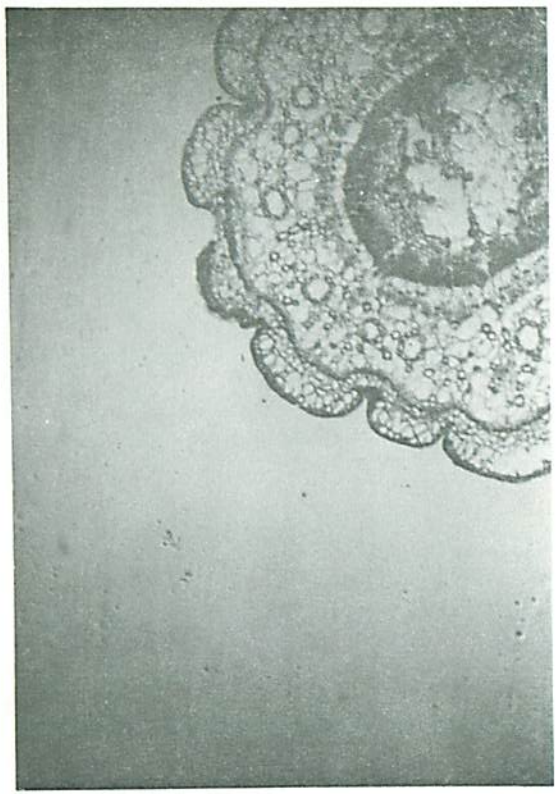
(c) x 400

Fig. 9. Penetration and movement of fenitrothion - C^{14} through balsam fir needle. Transverse sections showing distribution and localization of silver grains in (a) cuticle, (b) pallisade cells and (c) vascular (xylem) bundles of needles. Needles treated with radioactive fenitrothion for 16 days.

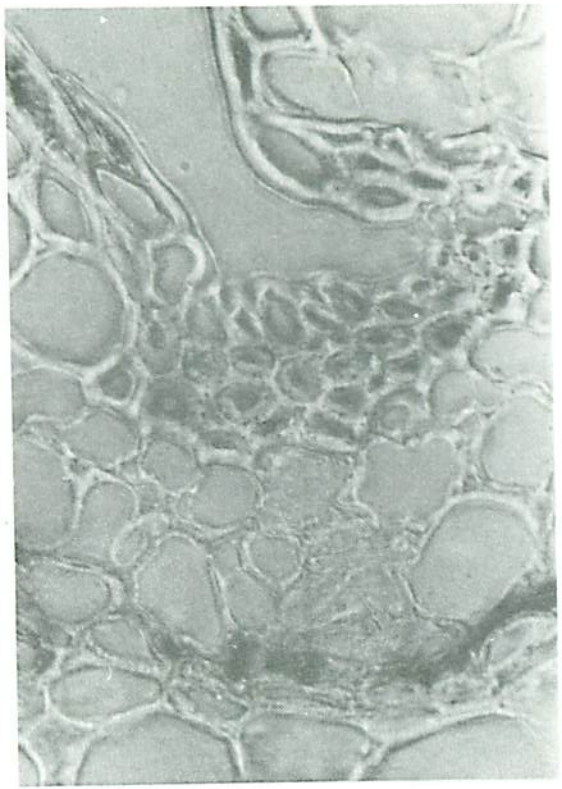


(a) (b) (c)

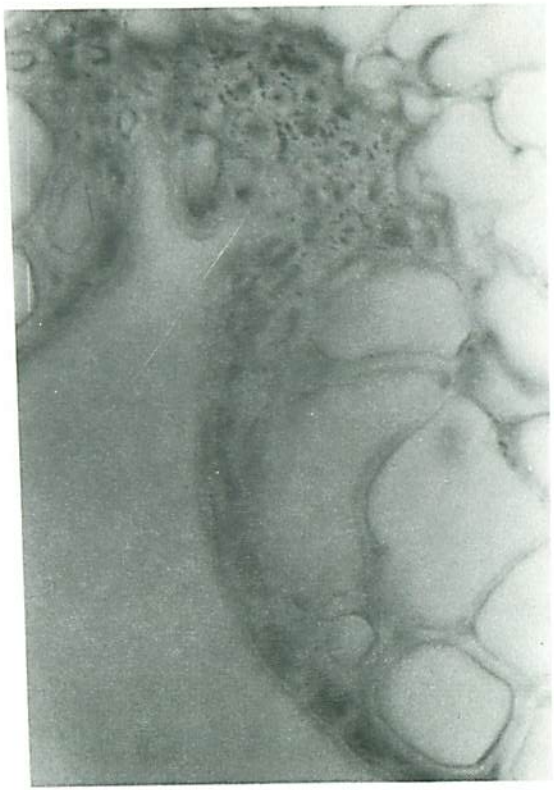
Fig. 10. Localization of activity in vascular system of (a) spruce and (b) and (c), balsam fir stem treated with fenitrothion for 21 days. Most of the silver granules are localized in xylem vessels (x 400).



(a) x 40



(b) x 400



(c) x 400

Fig. 11. Accumulation of radioactivity (silver grains) in the epi- and hypodermis regions of fascicular tissue of spruce rachis treated with fenitrothion C^{14}. Note the accumulation in the "groove".

SUMMARY AND CONCLUSIONS

A histoautoradiographic study to localize the residues of fenitrothion-C^{14} in balsam fir and white spruce was carried out. Because fenitrothion is soluble in many histological solvents, the standard methods of paraffin and carbowax embedding did not prove satisfactory. Instead, the optimal cutting temperature (O.C.T.) method employing a cryostat was found to be the most suitable, and for coniferous tissues a 10% glycerol solution was found to be the most appropriate cryoprotective agent. Tissues pretreated with this anti-freeze yielded reproduceable sections of good quality.

The radioactivity (silver grains) persists in the cuticle, epidermis, and hypodermis, of needles of both species for 21 days and some movement into the inner tissues (mesoderm and vascular system) also takes place. Silver grains were also found in xylem vessels of balsam fir and spruce stems as well as in the epi- and hypodermal regions of the fascicular tissues.

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BIBLIOGRAPHY

Annon, (1955) Con-Rad/Joftes Fluid Emulsion System for Radiography of Biological Specimens. Control for Radiation Inc. Cambridge Mass. Tech. Bulletin 23 pp.

Appleton, T.C. (1964). Autoradiography of Soluble Compounds. J. Roy Micros. Soc. 83 : 277.

Fettes, J.J. (1968). Chemical control of forest insects by aircraft. Pulp and Paper Mag. of Canada 69 (18) : 90.

Fischer, H.A. and G. Werner (1971). Autoradiography N.Y., Walter De Gruyter, 199 pp.

Hallett, D., P. Weinberger, and R. Prasad (1973). A preliminary study on the Fate of Fenitrothion in Forest Seeds. I. Determination of residues and their effects on amino acid metabolism. Inf. Rept. C.F.S. CC-X-50, 22 pp.

Humason, G.L. (1962). Animal Tissue Techniques. San Francisco, W.H. Freeman and Co. 468 pp.

Jensen, W.A. (1962). Botanical Histochemistry. San Francisco, W.H. Freeman and Co. 408 pp.

Knox, R.B. (1970). Freeze Sectioning of Plant Tissues, Stain Technol. 45, 265-272.

Martin, J.T. and B.E. Juniper (1970). The Cuticle of Plants. Edward Arnold Ltd., 347 pp.

Morris, O.N. (1971). Metabolic Changes in Diseased Insects. IV. Radio-autographic studies on protein changes in NPD and NIV infections. J. Invert. Pathology 18(2) 101-206.

Norris, R.F. (1974). Penetration of 2, 4-D in Relation to Cuticle Thickness. Am. Jr. Bot. 61(1) 74-79.

Pease, D.C. (1966). The preservation of unfixed cytological detail by dehydration with "inert" agents, J. Ultrastruct Res. 14: 356-78.

_____ (1967). Eutectic ethylene glycol and pure propylene glycol as media for dehydration of frozen tissues. J. Ultra Res. 21: 75-97.

Prasad, R., K.M.S. Sundaram and W.N. Yule (1974). Persistence & Fate of Fenitrothion in Forest Trees. Can. For. Serv. Inf. Rept. (in preparation).

Randall, A.P. (1970). Some Recent Aspects of Forest Insect Control Rept. NE Aerial Appl. Conf. Cornell N.Y. 91.

Yule, W.N. and J.R. Duffy (1972). The Persistence and Fate of Fenitrothion Insecticide in a Forest Environment. Bull. Env. Contam. and Toxicol. 8(1):10.

APPENDIX