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# Two rapid fluorescence procedures for the detection of some thio pungent compounds in plant tissues

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**Summary**

Two rapid flourescence procedures are described for detecting sulphydryl, disulphide and isothiocyanate groups of scented and pungent principles present in the vacuolar sap of onion, garlic and cabbage. To localize compounds containing sulphydryl groups, fresh or fixed frozen sections of the plants were treated with mercurochrome. After the fluorochromization, strongly-positive sulphydryl sites emitted an intense orange-red fluorescence, while weakly-positive sites emitted a distinctive red-brown fluorescence. Disulphide groups were detected by first reducing with thioglycolic acid to thiol groups before treating with mercurochrome. To effect isothiocyanate localization, frozen sections were exposed to ammonia: isothiocyanates were converted to thioureas and the engendered amino groups were revealed with fluorescamine.

## Introduction

From a theoretical point of view, it should be possible to localize, by fluorescence microscopy, a wide variety of compounds in plant tissues having nutritional, pharmacological or toxicological significance. A number of scented and pungent principles of plants, used pharmacologically and as condiments, consist of a heterogeneous mixture of sulphur-containing compounds (Macleod & Macleod, 1970). Although many methods for the detection of sulphated substances in animal tissues are available in the literature (Pearse, 1968), only a few procedures can be used in the localization of plant thio principles because of the high ethanolic solubility of these compounds and the shortage of highly-sensitive methods. The present study was undertaken to explore the feasibility of using direct fluorochromy and induced fluorescence for localizing thio pungent principles in fixed and fresh plant tissues.

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## Materials and methods

Pieces of onion and garlic bulbs and portions of cabbage leaves, known to contain thio pungent principles, were employed in this study. The specimens were either used fresh or fixed in formaldehyde gas at 80° C for 1 h in a closed vessel (1000 ml) containing 5 g paraformaldehyde previously equilibrated in air with approximately 70% humidity. Ethanolic fixation was not employed because of the high solubility of thio principles in this solvent. Theoretically, formaldehyde fixation may provoke some complications inducing secondary fluorescence (Ganter & Jollés, 1970), but this disadvantage is minimized by the insignificant extraction of thio principles from tissues. For the preparation of sections, fixed or unfixed, pieces of bulbs and leaves were immersed in water in gelatine and immediately frozen at −35° C (Chayen *et al.*, 1960). A cold microtome (Kryomat 2, Leitz) with methanol as the freezing fluid at −40° C, was used for freeze-cutting both longitudinal and cross-sections (20–30 μm).

### *Cytochemical reactions*

To localize thio compounds containing sulphydryl and disulphide groups, such as *n*-propyl sulphides, methyl sulphides, allyl sulphides and allyl propyl sulphides, the sections were sprayed with a 0.02% aqueous solution of mercurochrome (dibromohydroxymercurifluorescein, disodium salt) and, after a few seconds, washed briefly in distilled water and mounted in water or in glycerol and observed directly. The mercurial compound reacts with sulphydryl groups (Pearse, 1968; Cowden & Curtis, 1970; Pandya & Machwe, 1977) and keeps the sulphated principles *in situ*, because of the large size of the macromolecular complex formed by the organomercurial-sulphated compound. Mercurochrome was chosen because it is soluble in water and is simple to apply for the localization of sulphydryl groups.

After reduction of disulphide to thiol groups with thioglycolic acid (Lillie, 1965), the mercurochrome test was again carried out to localize both the new sulphydryl groups and those previously present. The presence of principles containing disulphide groups was evaluated by comparing specimens before and after the reduction treatment.

To demonstrate that the fluorescence emission was only due to thio compounds, two kinds of control experiments were carried out. (a) A series of reactions by blocking the sulphydryl groups with iodoacetate or N-maleimide (Ganter & Jollés, 1970). This procedure was also repeated to evaluate the sulphydryl groups produced from the conversion of disulphide to sulphydryl groups. (b) A series of experiments based on the extraction of the sulphur-containing principles with warm ethanol (40° C) for 30 min. It is well known that thio principles are very soluble in ethanol (Kjaer, 1961); therefore, it is possible to extract them before fluorochromization with mercurochrome. In this way, by comparing the specimens treated only with mercurochrome with those treated with ethanol and then fluorochromized, it is possible to check the presence of the thio principles in the cell. Theoretically, this procedure is not specific because the ethanolic treatment results in the extraction of several compounds from plant cells. From a practical point of view, however, the absence of fluorescence emission in the vacuole of the parenchymatous cells in these specimens treated with ethanol and fluorochromized shows that thio compounds were extracted. In fact, the typical scent was emitted by the ethanol used in the treatment. In addition, when the extraction was carried out after fluorochromization and the ethanol used was placed on a chromatographic sheet, the spot formed contained the thiol-mercurochrome complex as demonstrated by using a ultraviolet lamp at 435 nm.

In order to localize compounds containing the isothiocyanate groups, the sections were pre-treated with ammonia gas. In this way, isothiocyanates were converted to thioureas (Devani *et al.*, 1973) and the new amino groups, so engendered, were revealed with

fluorescamine, a very sensitive test for demonstrating amino groups by induced fluorescence (Håkanson *et al.*, 1974; Bruni *et al.*, 1976). The ammoniated sections were sprayed with fluorescamine (Bruni *et al.*, 1977), mounted in a phosphate buffer (0.2 M, pH 8.0) and observed directly.

The ammonia vapour–fluorescamine reaction induces fluorescence only when isothiocyanates are free but not when they are in a glycosidic form. When unfixed sections were cut, the hydrolytic enzymes contained in particular cells were released, permitting the hydrolysis of the thioglycosides to free isothiocyanates. The histochemical test reaches an optimum after 10–15 min, a time which ensures the completion of enzymic hydrolysis. Controls were made as follows: (a) on untreated sections, to observe the autofluorescence; (b) on ammoniated sections, to observe the fluorescence which might be induced by ammonia; (c) on sections treated with fluorescamine without ammonia, to observe the fluorescence induced by fluorescamine; (d) on sections treated with ammonia and fluorescamine and processed through warm ethanol (40° C, 30 min), to observe if the fluorescence was destroyed by ethanolic extraction; (e) on sections treated with ethanol and then fluorochromized by the ammonia-fluorescamine reaction, to observe if the fluorescence was present after ethanolic extraction.

*Fluorescence microscopy*

The slides were viewed with a Zeiss Photomicroscope II equipped with an incident fluorescence condenser (F1, II), and an XBO-75 xenon arc source. For the fluorescamine test, a Schott UG1 was used as the primary filter and a Schott OG4 as the secondary filter. For mercurochrome detection, a 460 nm dichroic mirror, a series of barrier filters (47, 50) and UG2 primary filter were employed. Photographic prints were made from HP5/135 Ilford film developed in a Microfile developer.

## Results and discussion

Microscopic examination of fixed and unfixed sections, fluorochromized by the organomercurial compound, indicates the presence of sulphydryl-containing compounds in plant tissues. The observed fluorescence could be due to substances containing sulphydryl groups which are different from the active principles, such as proteins, peptides, aminoacids, etc. The fluorescence of these compounds, however, is localized at the cytoplasmic and nuclear level and resistant to ethanolic extraction. On the contrary, the fluorescence at the vacuolar level is entirely destroyed by alcohol treatment, a fact which is consistent with the presence of thio principles. Before ethanolic extraction, the sites emitting an intense orange-red fluorescence indicate the presence of a large amount of thio principles, while those showing a distinctive red-brown fluorescence, signify that the compounds are present in a limited quantity only. The green fluorescence appearing in the sections may be attributed to unreactive sites and to the typical autofluorescence of the plant cell. In the formaldehyde-fixed material, green fluorescence is induced by the fixative. This behaviour is well known (Pearse, 1968) and can limit the examination of specimens, but in our case, it enhanced the contrast because the positive sites were stained in a different colour.

In all the specimens studied, when sulphydryl-containing compounds were present in small amounts, a weak but significant fluorescence was visible. On the contrary, when sulphydryl plus disulphide groups were tested, the fluorescence efficiency was very strong. The control experiments were consistently negative: when sections were pre-treated with a thiol-blocking reagent and then subjected to the standard mercurochrome procedure, the normally positive sites emitted a green fluorescence, whereas unreactive sites retained their original fluorescence colour. The fluorescence of the positive sites was destroyed by warm ethanol.

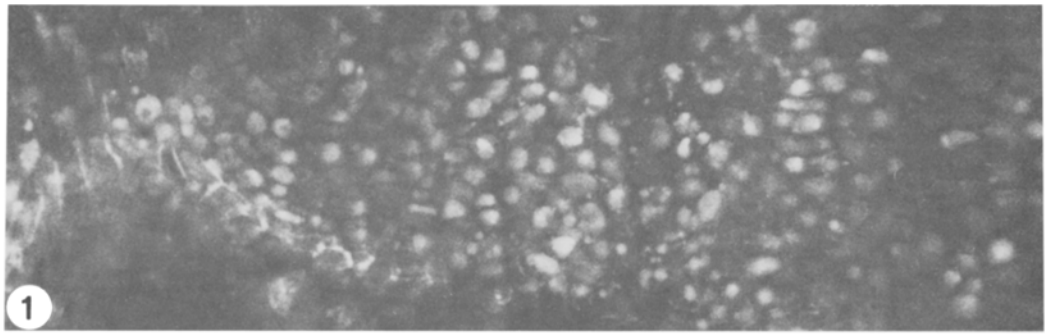
As regards the detection of isothiocyanate-containing principles after fluorescamine treatment, the positive sites exhibit an intense green fluorescence at vacuolar level. The detection of the fluorescence induced by the isothiocyanate-fluorescamine reaction is facilitated by the constant comparison between non-fluorochromized and fluorescamine-treated sections. In this way, a sharp contrast between positive and negative sites can be readily obtained. An additional control by ethanolic extraction confirmed the observations, giving indirect evidence of topochemical detection. Contrary to sulphydryl and disulphide group localization, fixation must be avoided in isothiocyanate detection. In fact, the fixative prevents the enzymic hydrolysis of the glycosidic form (unreactive) to the free isothiocyanates (reactive).

The results obtained confirm the data collected from other authors who used chemical analysis in plant tissues and organs (Rousseau, 1940; Kjaer, 1961; Josefsson, 1967; Greenhalgh & Mitchell, 1976), and indicate that the isothiocyanates are particularly abundant in cabbage, whereas they are present in small quantities in garlic and onion bulbs. More specifically, in cabbage, a green positive fluorescence is evident in parenchymatous tissues (Fig. 1) and in cells surrounding vascular bundles. In the garlic mesophyll, the cells containing thio principles are, instead, randomly arranged, but may be found primarily around the vascular strands (Fig. 2). The epidermis is particularly sensitive to the proposed methods. In garlic, the epidermis shows a mosaic-like image due to the different amounts of thio compounds in the cells (Fig. 3), whereas in both garlic and onion bulbs, the younger cataphylls are more fluorescent than the older ones.

**Fig. 1.** Transverse fresh section of cabbage leaf in which isothiocyanates are present in the parenchymatous cells. Their distribution, as revealed by ammonia vapour-fluorescamine reaction, is ubiquitous. × 220.

**Fig. 2.** Transverse fresh section of a cataphyll of onion. Cells surrounding the vascular bundles after mercurochrome treatment show an intense orange fluorescence due to the thio principles. × 495.

**Fig. 3.** Fixed section of garlic epidermis fluorochromized by mercurochrome after thioglycolic acid treatment. The polychromatic 'patchwork-like' image is related to the different amounts in sulphydryl and disulphide group-containing principles. × 495.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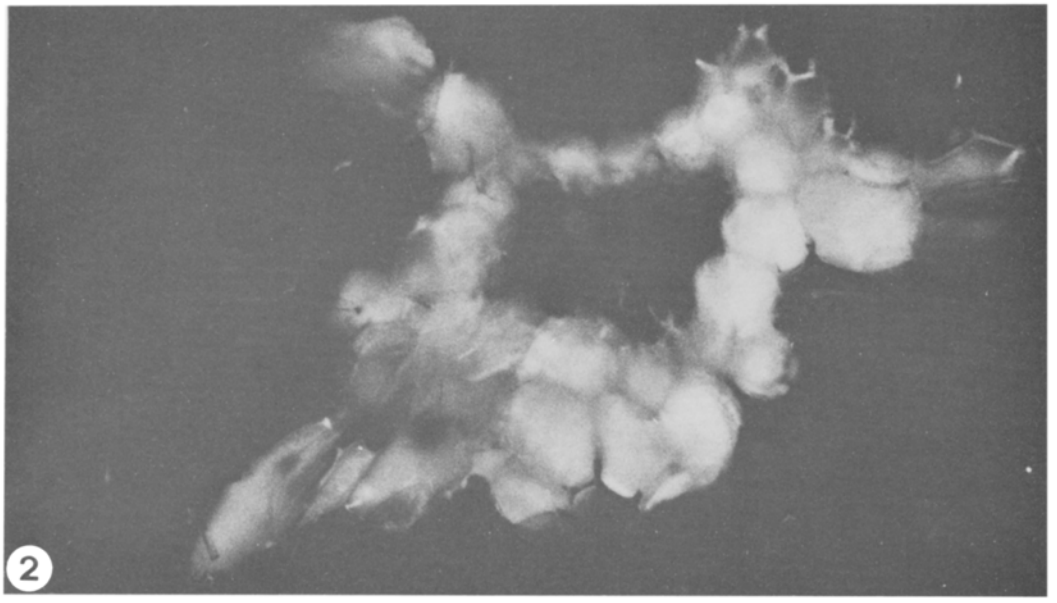
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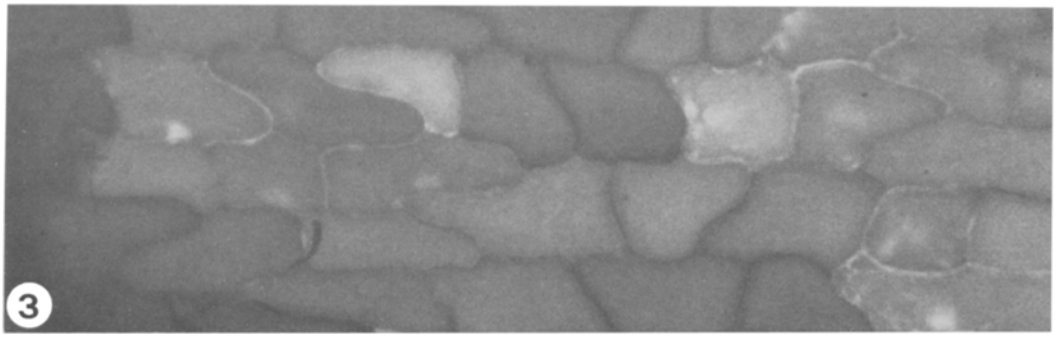
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## Conclusion

These topochemical methods are capable of localizing active principles and can shed some light on the histochemical distribution of thio pungent compounds of *Liliaceae* and *Cruciferae* plants. Furthermore, they can furnish some indication about their biosynthesis during tissue differentiation. The advantages of examining plant sections processed with the mercurochrome test can be summarized as follows: (a) good sensitivity; (b) a clear difference is evident between reactive and unreactive sites; (c) the ability to carry out rapid specific controls by ethanolic extraction. These advantages offset possible disadvantages, such as the necessity of adequate training in the use of the cold microtome and the need to operate quickly because of fluorescence fading, particularly when the sections are to be photographed. However, it is possible to overcome some of the difficulties of photography by making use of the hypersensitization of films, such as the one proposed by Vanderploeg *et al.* (1976).

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