Brief Review on the Application of Histochemical Methods in Different Aspects of Plant Research

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Abstract: Histochemical methods are employed in the identification, density of accumulation and distribution of chemical compounds within biological cells and tissues in different organs under microscopes using the color-stain-reaction technique and photographic recording. These include the preparation of fixed variably stained specimens and then the examination under the microscopic devices. In immuno-histochemistry, antibodies are mainly used to visualize antigens in sections of tissue under either the light or the electron microscope. Histochemistry reperesent elusive documents to be used for identifying cellular chemical processes, cellular traffics, metabolite sorting, and in investigating several diseases, mainly related to cancerous activities. It is successfully applied in gene expression studies, detection and localization of cellular components of active cell constituents such as proteins, carbohydrates, lipids, nucleic acids, and a range of ionic elements occurring in the cell solutions, in addition to identifying the characterization of secretory structures and the chemical nature of the secreted compounds. Ion homeostasis could be assessed, while constituents of the signal transduction cascades could be identified through metabolic amplification. The methods played a role in describing and tracing the ultrastructure development during different plant growth stages so as the genetic bases of plant physiological and biochemical processes could be further elucidated. The penetration process and defense reactions (hypersensitive response, oxidative burst and cell wall fortification) of various organisms could be studied histochemically. This help in comparing the resistant and susceptible plant cell lines. It also assists in indicating mechanism of pathogen invasion and hypersensitive responses. To examine the supposed pollution and contamination in a given location, histochemical methods are efficient in detecting the existence of certain injurious metals via samples of plants taken from the inhabiting area. It is however concluded that such methods are proved as good tool to be rapidly and efficiently employed in different vital aspects of biological research.

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I-Introduction

Histochemistry is devoted to study the identification and distribution of chemical compounds within and between biological cells, using stains, indicators and light and electron microscopy (Wick, 2012). To understand the definitions of histochemistry, it may be helpful to recall the definitions of histology as the microscopic study of the structure of biological cells and tissues, whereas, chemistry is the science of matter and the changes that occur between and via different chemical reactions. Thus, leading to the changes occur in molecules and cell components.

While histochemistry has broad interface with life sciences as one of the most objective methods in biology and medical research, botany was the principal scientific discipline in which such technique was evolved. While botanists retained a basic interest in the cellular chemical processes that were illumined by histochemistry, zoology-oriented histologists and histochemists used microscopy and staining technique primarily to further the development of microanatomy, taxonomy and nosology (Wick, 2012).

The methods are found to be efficient tools for analyzing, localization and distribution of molecules in cells and tissues. This include proteins, carbohydrates, lipids, nucleic acids and a range of ionic elements occurring in cell solutions. These methods are essential in pathologic diagnosis (Kiernan, 1999). Histochemists pioneered the use of small-molecule cellular stains, labeled molecules such as antibodies, and enzyme-mediated detection and signal amplification (Kiernan, 2008).

The technique is also employed to study time course of deposition and distribution of major storage compounds such as protein, lipid, starch, phytin, and minerals such as calcium, potassium and iron in rice grains (Krishnan *et al*., 2003; Krishnan and Dayanandan, 2003). Immunohistochemistry utilizes antibodies to visualize antigens in sections of tissue under the microscope in conjunction with light and /or electron microscopic examination. Light microscopy often possesses sufficient resolution to determine the distribution of an antigen between tissues and cell types (Famiani *et al*., 2000). Therefore, immunohistochemistry provided valuable information

about how the metabolism is compartmentalized between different tissues in plant structures (Walker *et al*., 2001).

II - Material and Methods:

1.Preparation of the plant tissues and fixation:

The plant tissue under investigation is collected, washed thoroughly under current water, cut (segments of 0.5-1cm) and preserved in fixative solutions for at least 48hrs.

The universal fixative solutions are formalin, acetic acid and alcohol (FAA). Chrom-acetic solution (for fresh algae and for fungi), Carnoy's solution (for chromosomes) and Erliki'sfluid (for mitochondria) are also used (Johansen 1940; Jensen 1962).

2.Tissue dehydration, paraffin infiltration and sectioning:

Following fixation, tissue samples passed through serial concentrations of dehydration solutions of ethanol and absolute alcohol. The dehydrated sections are then immersed in a mixture of absolute ethanol and xylene; followed by embedding in paraffin wax Uniform sections are prepared using microtome. The sections are affixed to slides using the Haupt's adhesive, which contain gelatin and glycerin. Before staining, the wax is to be dissolved by passing slides through xylene followed by a mixture of xylene and absolute ethanol. The sections are then transferred to a successive down series dilutions of ethanol from absolute ethanol. The methods are described in detail by **Johansen**, 1940; Jensen, 1962 and recently by (El-Awadi 2001).

3.Staining:

Table (1) illustrates some stains that are commonly used in histochemical tests.

Table 1. Stains and the cell structures` color reactions under the microscope (Jensen, 1962; O'Brien and McCully, 1981).

Stain	Investigated materials or tissues	Colour
Safranin-fast green	Cuticle, lignin, chromosome, nuclei	Red
Safranin-aniline blue	Gymnosperm	Blue
Tannic acid ferric chloride-safranin	Stem and root apical region Cuticle, lignin, chromosome, nuclei, cell wall	Red
Heidenhain's iron haematoxylin-orange G	Cytoplasmic particles and cell wall	Light orange
	Chromosome nuclei, plastids mitochondria	Dark blue
Delafield's haematoxylin	Cytoplasmic particles (chromosome, nuclei, plastids, mitochondria)	Deep blue black
	Cytoplasm	Blue gray
Toludine blue 0	Lignin	Blue green to turquoise
	Other phenols	Bright blue to dark violet
	Pectin and other celluloses	Pink to reddish
Phloroglucinol- HCl	Lignin	Red violet

4.Mounting the cover slips:

After staining, the tissue is dehydrated, and a cover slip is mounted over it. The cover slip makes the preparation permanent. The cover slip is held in place with a mounting medium. The medium should have the same refractive index as the glass index and should not affect the stained tissue. The traditional mounting medium is Canada balsam (Johansen, 1940; Jensen, 1962).

5.Microscopic Examination:

The prepared stained sections can be examined under the as Light Microscope, Electron Microscope, Stereoscopic Microscope and Fluorescent Microscope.

Fluorescent Microscope

A fluorescent microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic and / or inorganic substances (Lakowicz, 2006). It uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescent microscope, or a more complicated design such as a confocal microscope.

Fluorescence

Fluorescence is the emission of light by a substance that absorb light or other electromagnetic radiation. The emitted light usually has a longer wavelength, and therefore lower energy than the absorbed radiation. However, when the absorbed electromagnetic radiation is intense, it is possible for one electron to absorb two photons. Such two-photon absorption can lead to emission of radiation having a shorter wavelength than the absorbed radiation. The emitted radiation may also be of the same wavelength as the absorbed radiation, termed "resonance fluorescence (Skoog *et al*., 1997). The color indications of the autofluorescence of unstained and stained tissues (with fluorescent stains) under different illumination light sources are shown in Table (2).

Table 2. Colour indications of the autofluorescence of unstained and stained tissues (with fluorescent stains) under different illumination light sources

Stains	**Light**	**Fluorescent color**	**Materials**	**Reference**
Unstained plant sections	Ultraviolet (UV -2A)	Bright blue to whitish	Lignins and/or phenolics	Brammall and Higgins (1988); M El-Awadi 200…thesis
		Yellowish to brownish	Condensed tannins	
	Blue (B-2A)	Bright yellow	Lignins and/or phenolics	
	Green (G-2A)	Bright red	Phenolics	
Fluorol yellow 088	Ultraviolet (UV-2A)	Bright yellow	Lipid	Brundett *et al*. (1991)
Sudan III&IV	Blue (B-2A)	Bright red	Suberins	O'Brien and McCully (1981)

III-Examples of the Application of Histochemistry in Plant Research.

1. In gene expression

Green fluorescent protein (GFP) from the Jellyfish *Aequorea victoria* and its homologs from diverse marine animals are widely used as universal genetically encoded fluorescent labels (Giepmans et al, 2006).

Efforts on identification and development of fluorescent proteins with novel characteristics and enhanced properties, led to a powerful toolkit for visualization of structural organization and dynamic processes in living cells and organisms. The diversity of available fluorescent proteins covers the entire visible spectrum, providing numerous alternative possibilities for multicolor labeling and studies of protein interactions (Chudakov *et al*., 2010).

Sidorov *et al.* (1999) described a reproducible plastid transformation system for potato and regeneration of plants with uniformly transformed plastids. Plastid-expressed green fluorescent protein was used as a visual marker for identification of plastid transformants at the early stage of selection and shoot regeneration (Fig. 1).

The establishment of a plastid transformation system in potato offers new possibilities for genetic improvement of this important crop.

The detection of plant transformation (using β-glucuronidase; GUS assay in *Lilium* are carried out via the application of histochemical methods.

β-glucuronidase (GUS) assay is used to assess transient expression of the GUS gene using 5-bromo-4-chloro-3-indolyl β D-glucuronide (X-Gluc) as the substrate.

Six days after co-cultivation, samples of 0.1g of callus collected from each treatment are subjected to transient histochemical GUS assay.

The transformation efficiency of calli are evaluated by counting the number of blue spots, using stereomicroscope, showing GUS enzyme activity on each callus sample (Stomp, 1992). The methods / technique are described by Azadi *et al*., (2010) as shown in Fig. (2).

The use of the histochemical methods in iron and ferritin gene expression in transgenic indica rice (*Oryza sativa* L. cv Pusa Basmati) proved as efficient in such an investigation. Perl's Prussian blue staining of transgenic rice grain sections show distribution of iron accumulation (blue compound of ferric ferrocyanide) throughout the alureone and subaleurone layers and in the central region of the starchy endosperm (Fig. 3-b). Whereas, in the nontransgenic grains, blue colour formation indicating iron accumulation was restricted to the aleurone layer and the intensity of color was also very low (Fig. 3-a).

Transverse section of the transgenic rice grains indicated the high iron accumulation in embryo as well as in the endosperm (Fig. 3-d), in comparison to the nontransgenic ones. In the latter, iron appeared restricted to the embryo and aleurone layer in which the intensity of color detected in the embryo was very low (Fig. 3-c). This histochemical analysis of iron in rice specifically showed temporal and spatial deposition of storage iron (Sivaprakash *et al.,* 2006).

Advances in histochemistry and cytochemistry made it possible to retrieve quantitative data from 2D and 3D microscopic images. In this way, valid quantitative results can be regenerated (e.g. gene expression data at the mRNA, protein and activity levels) from microscopic images in relation to structures in cells, tissues and organs in 2D and 3D. Volumes, areas, lengths and numbers of cells and tissues can be calculated and related to these gene expression data while preserving the 2D and 3D morphology (Chieco *et al*., 2013).

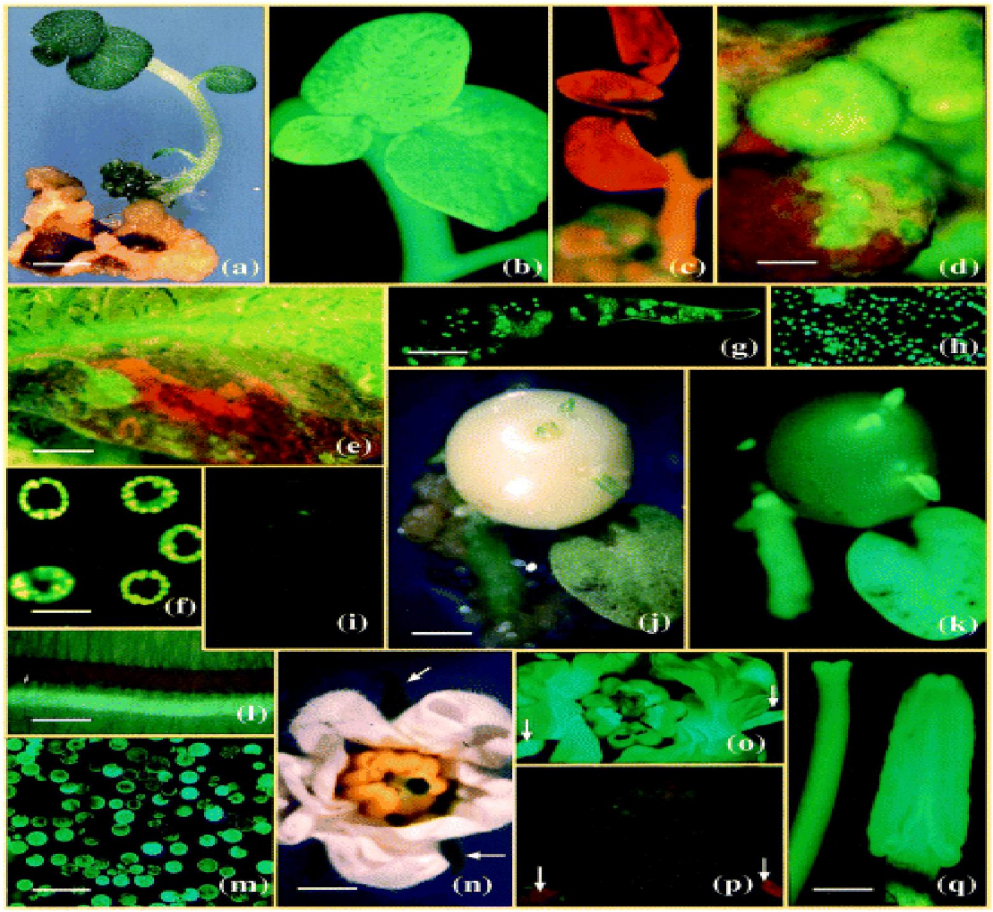


Fig. 1. GFP in plastid transformants of potato (Sidorov *et al*., 1999).
(a) Appearance of spectinomycin resistant regenerate under visible day light condition (bar = 8 mm). (b) GFP fluorescence of transformed and (c) autofluorescence of non-transformed regenerants excited by blue light. (d) Chimeric callus (bar = 0.3 mm) and (e) leaf segment (bar = 0.8 mm) illuminated with blue light. (f) GFP expression in chloroplasts of stomatal guard cells of leaf epidermis (bar = 38 µm). (g) Expression of GFP in plastids of trichome cells (bar = 60 µm). (h) Fluorescence of freshly isolated mesophyll chloroplasts under blue light. (i) Appearance of wild-type microtuber under blue light. (j, k) Microtubers of transformed plants under visible and blue light, respectively (bar = 3 mm). (l) Red autofluorescence (top) and green GFP fluorescence (bottom) of roots from non-transformed and transformed plants (bar = 0.4 mm). (m) Isolated mesophyll protoplasts in blue light (bar = 220 µm). (n) Transplastomic potato flower under visible (bar = 3.6 mm) and (o) blue light. (p) Appearance of wild-type flower under blue light. Arrows in (n, o, p) indicate the sepals. (q) Detection of GFP fluorescence in pistil and anther, excited by blue light (bar = 1.6 mm).

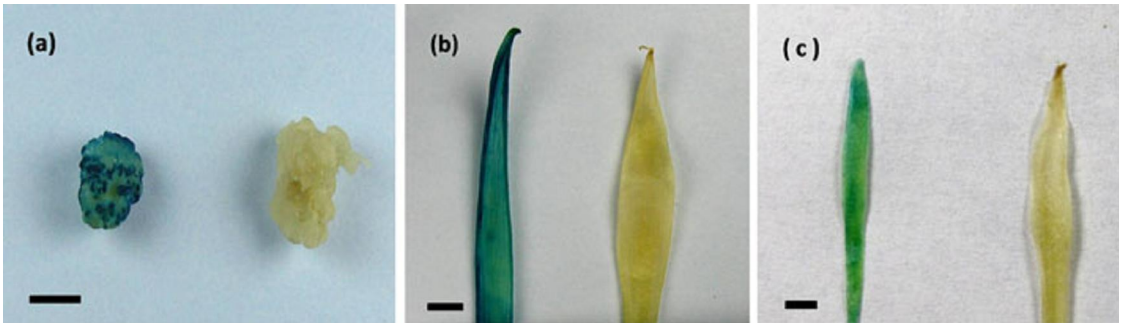


Fig.2. Histochemical β-glucuronidase (GUS) assay of transgenic plants. Stable GUS expression on calli (a), leaf of *Lilium* x *formolongi* 'Akasu' plants (b), and leaf of 'Acapulco' (c). Transgenic plant (left) and non-transformed plant (right), respectively (Azadi *et al.,* 2010).

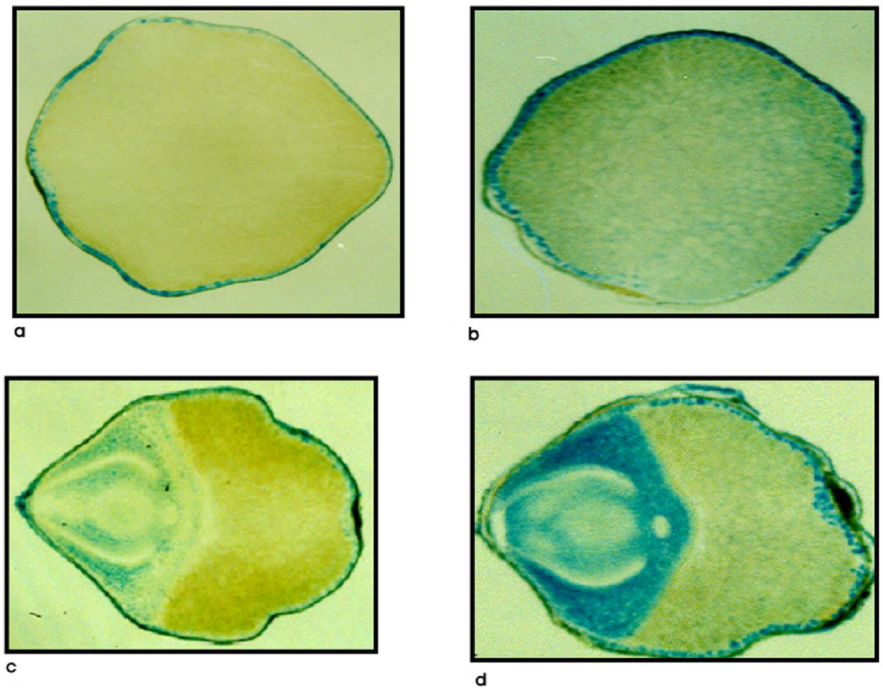


Fig. 3. Transverse section of mature nontransformed (a, c) and transgenic (b, d) rice grains. Blue colour indicates the presence of iron. In control, iron is the restricted to aleurone and embryo, and is not found in the endosperm. In transgenic rice grain, in addition to aleurone and embryo, iron is also strongly present in the endosperm cells. Higher accumulation of iron in embryo of transgenic rice, is also shown by intensity of color compared to control (Sivaprakash *et al*., 2006)**.**

2.In detection and localization of lignin, phenolics, lipids, suberin, alkaloids and tannins in certain plants.

In a study of leaf structure and secretory activity of *Ecballium elaterium* (Fig. 4)**;** a hairy pharmaceutical perennial plant; histochemical method was applied. The amphistomatic leaf showed a characteristic structure due to special cells supporting the conductive bundles, a remarkable shortage of mechanical tissue, and the existence of pectin strands between mesophyll cells. The secreting activity is limited mostly to secretary hairs. Such a structural character points to a remarkable strategy of such a species coping with stress conditions of its habitat (Nikolaos *et al*., 2011).

In such a study, unsaturated lipids, sesquiterpenes, flavonoids, terpene containing steroid, alkaloids, tannins, catechol tannins, polyphenols and other phenolic compounds, monoterpene phenols and phenolic tannin precursors were identified and localized within the cells.

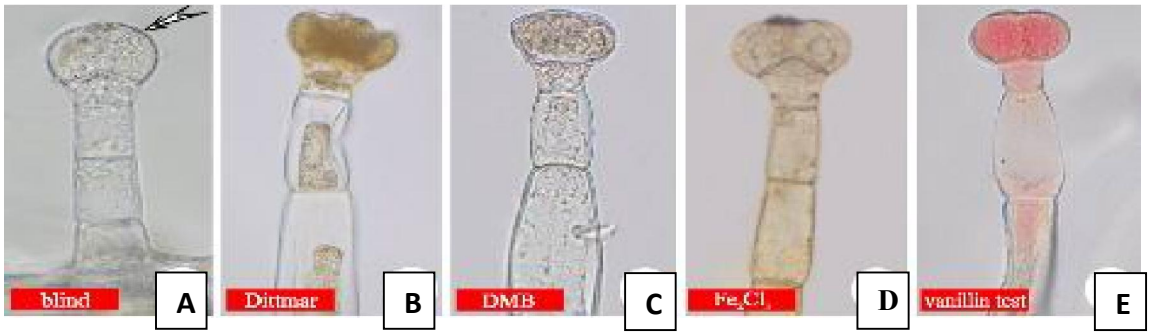
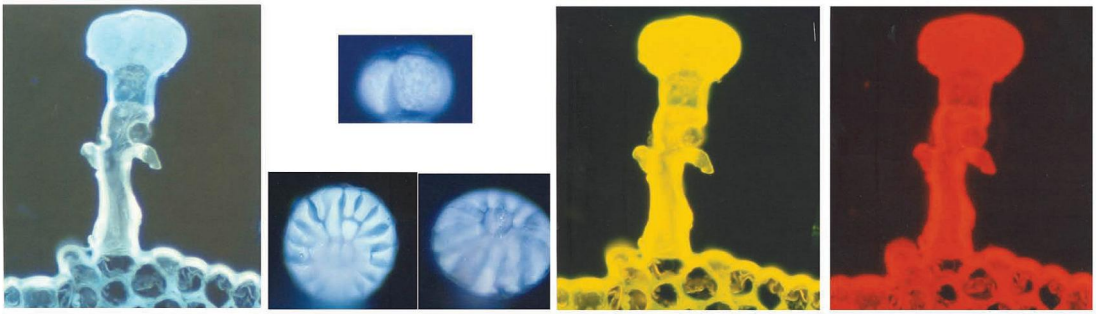


Fig. 4. Histochemical investigation of *Ecballium elaterium* secretive hairs (Nikolaos *et al*., 2011).
(A) Blind, no treatment. The arrow points at the head-cells, (B)Dittmar reagent for alkaloids; (C) DMB (DiMethoxy Benzaldehyde or veratralaldehyde) for phenolic tannin precursors; (D) Ferric chloride for polyphenols; (E)Alcoholic vanillin/ HCl (vanillin test) for phenolic compounds.

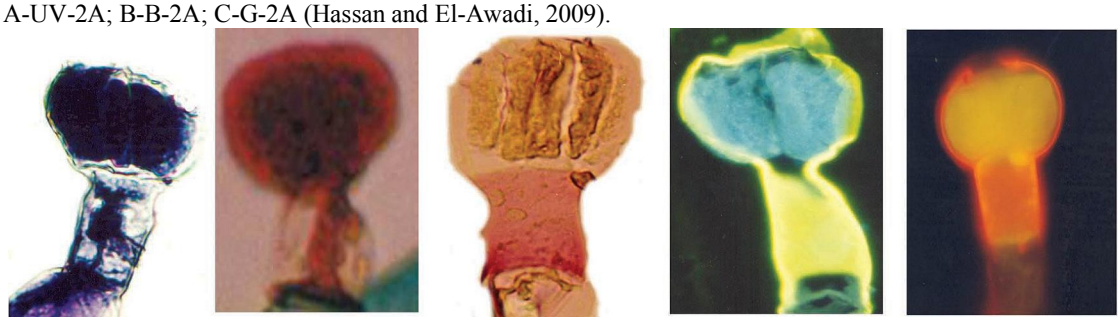
Corresponding results indicated the existence of lignin, phenolics, lipid and suberin materials in the glandular trichomes of the parasitic plant broomrape. The phenolic substances were detected in the neck cell and gland secreted cells by autofluorescence and histochemical tests (Hassan and El-Awadi, 2009). However, secreting cells inside the gland had emitted the whitish autofluorescence (UV-2A) of lignin and phenolic substances as shown in Fig. (5-a).

The histochemical light - stain reaction tests revealed the presence of lignin (red, double stain), phenolics (blue, toludine blue O), lipid (yellow, fluoroll yellow 088 or red, fat red 7B) and suberin (red, mixed Sudan III&IV). These substances were located in the outer layer of trichomes and in the neck cell. In secreting cells of the examined gland, the phenolic substances were detected by the toludine blue O and the double stains (Fig. 5-b)



UV-2A B-B-2A G-2A

Fig. 5-a. Autofluorescence of non-stained sections showing the light-colour reaction of broomrape glandular trichome under A-UV-2A; B-B-2A; C-G-2A (Hassan and El-Awadi, 2009).



A **B** **C** **D** **E**

Fig. 5-b. Histochemical tests showing the chemical composition of broomrape plant glandular trichomes. A, stained with toludine blue O under visible light; B, Stained with double stain under visible light; C, stained with fat red 7B under B-2A light; D, stained with fluorol yellow 088 under UV-2A light; E, stained with Sudan III & IV under B-2A light (Hassan and El-Awadi 2009).

Histochemical color-stain reaction methods applied in weedy *Amaranthus viridus* L. plant indicated highly subersized cuticle using sudan *III* & *TV* mixed stain under blue light illumination (red colour). Strong lignifications in outer layer appeared as red color and in between cortical cell (torques color) was detected by toludine blue o stain (Hassan *et al.,* 2003) as shown in Fig. (6).

3- **In the supposed defense mechanism actions:**

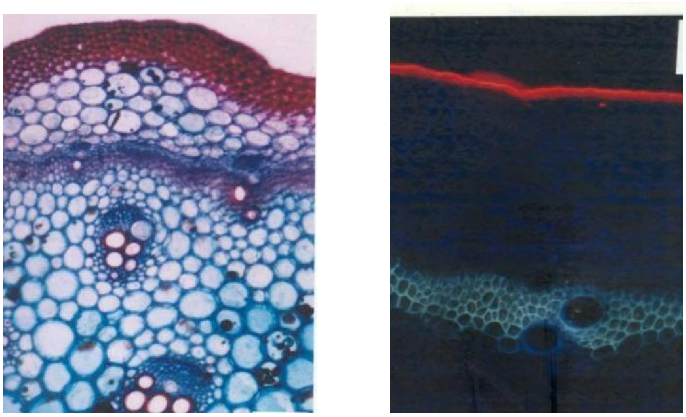
a-Parasitic plant host - root interaction

Wound responses in broomrape host root tissues` and in a host-pathogen interaction

Roots of different hosts of the holophrastic weed known as broomrape (*Orobanche spp.*) were examined histochemicaly for the occurrence of structural cellular barrier formation following wounding / penetration (Tables 3 and 4). Such barrier might function to impede the successful development of parasite haustorium interaction, i.e. as a self-defense mechanism.

In faba bean and white bean, brown deposits occurred in walls adjacent to the damaged cells of the epidermis, cortex and stele. Via stain reactions and colorations these deposits were detected as melanin. Additionally, walls bordering damaged site at the level of the endodermis and within the stele become suberized and lignified. In peas, which possesses a lignified hypodermis, the response was similar but lignin was also deposited in the walls of the endodermis and hypodermis adjacent to the wound. In sunflower, which possesses a suberized hypodermis, melanin was deposited in the hypodermis and lignin and suberin occurred within the stele. In all these broomrape host species

melanization conferred the modified cell wall many of the properties associated with lignified and suberized structures such as impermeability and resistance to chemical degradation (Brammall and Hassan, 1995).



A B

Fig. 6. *Amaranthus viridis* stem (Hassan *et al.,* 2003)
A: Toludine blue o stain under visible light and B: Sudan *III* & *TV* under blue light

Table 3. The distribution of lignin, suberin and melanin in uninjured tissues of faba bean, peas, white bean and sunflower (Brammall and Hassan, 1995)

Defense materials	**Faba bean**	**Peas**	**White bean**	**Sunflower**
Lignin	Xylem Phloem Endodermis Casparian band	Xylem Phloem Epidermis Outer cortical layer (Hypodermis) Endodermis Casparian band	Xylem Phloem	Xylem Phloem Casparian band
Suberin	Endodermal cells	Outer cortical layer (Hydridermis) Endodermis Casparian band	Endodermal cells	
Melanin	Epidermis Outer cortical layer	Epidermis Outer cortical layer	Epidermis Outer cortical layer	Epidermis

Table 4. The distribution of lignin, suberin and melanin in wound healing root tissues of faba bean, peas, white bean and sunflower (Brammall and Hassan, 1995)

	Faba bean	Peas	White bean	Sunflower
Lignin	Interior to endodermis	Interior to endodermis Epidermis Hypodermis	Interior to endodermis	Stele
Suberin	Endodermal cells	Hydridermis Endodermis Casparian band	Endodermal cells stele	Hydridermis (Outer cortical layer) Stele
Melanin	Epidermis cortical parenchyma stele	Epidermis Cortical parenchyma stele	Epidermis Cortical parenchyma stele	Epidermis Cortical parenchyma Stele

b-Host-pathogen interaction

In the Japanese birch (*Betula platyphylla* var. japonica, Tohoku), infection with a canker-rot fungus; the *Inonotus obliquus* IO-U1 strain. In intact wounded, and infected plantlets changes were observed morphologically in the treated portion. Phenolics first deposited at the cut margin. Subsequently the phenolic was detected in the vessels after 4h infection. Their deposition then extended to the xylem elements, the cortex, and to the pith with an increase in the infection period. The deposition of phenolics was extensive at 10 days post inoculation (dpi), when most of the cells were entirely filled with phenolics.

A necrophylactic periderm (NP) was formed at the junction of the original periderm with a layer of 2–4 new phellen cells at 30 dpi. Based on the results obtained, phenolics deposition and NP formation are considered to occur as infection - induced responses in Tohoku birch plantlets under infection with *Inonotus obliquus*, strain IO-U1 (Rahman *et al*., 2008).

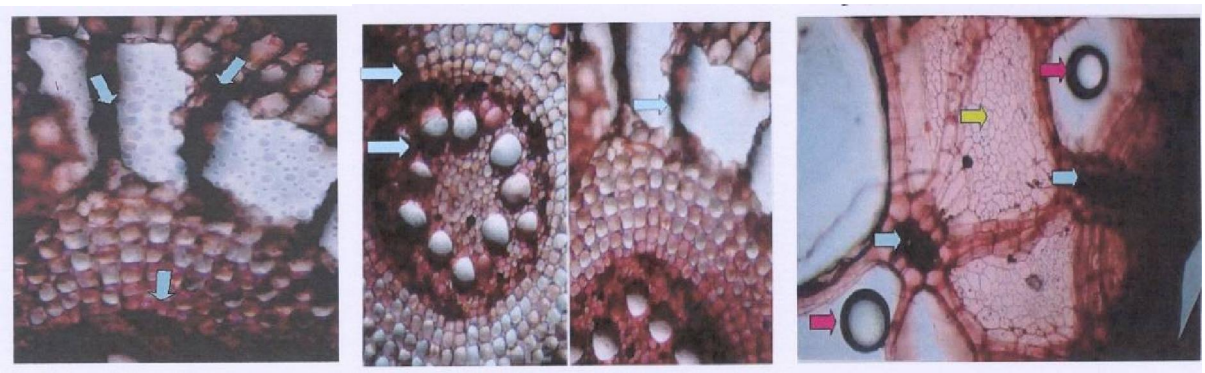
4-In detection of heavy metals,i.e. pollution and contamination:

a-Heavy metals` accumulation

Histochemical methods were employed in the detection of the heavy metals (Cd, Pb, Ni, Zn) and strontium, their distribution, accumulation, and translocation within the tissues of higher plants (Table 5). In this respect, detailed protocols of metal detection with metallochrome indicators dithizone (Cd, Pb), dimethylglyoxime (Ni), sodium rhodizonate (Sr), zincon (Zn), and fluorescent indicator Zinpyr_1 (Zn) by light and fluorescence microscopy were described (Seregin and Kozhevnikova, 2011).

In their study Warrier and Saroja (2008) investigated the occurrence of heavy metals and their accumulation in water hyacinth [*Eichhornia crassipes* (Mart.) Solms].

The histochemical staining examinations indicated the accumulation in the epidermis and vascular bundles of the roots and petiole. In the leaf sections the palisade tissues were deeply stained, showing the high accumulation of the metals within the leaves (Fig. 7).



A B C

Fig. 7. Histochemical investigation showing accumulation of heavy metals in different tissues of water hyacinth (Warrier and Saroja, 2008):
A: Cs of root showing translocation of heavy metals to the vascular bundles from the epidermis
B: Cs of root showing accumulation of heavy metals in the epidermal regions and translocation on the vascular bundles.
C: Cs of petiole of hyacinth
Absence of heavy metals accumulation with the petiole
Translocation of heavy metals through the vascular bundles of the petiole
Air bubbles

Table 5. Histochemical analysis for detecting distribution of Cd, Pb, Ni, Sr, and Zn in plant tissues

Reagent	Metal	Protocol of reagent preparation and color of produced complex/ fluorescence
Dithizone $C_{13}H_{12}N_4S$ (M*r* = 256.3) (metallochrome indicator)	Cd, Pb	Dithizone is dissolved (0.5 mg/ml) in a mixture of acetone and distilled water (3:1); 1–2 drops of glacial acetic acid are added to 6 ml of this solution in order to improve the sensitivity of reaction. Solution should be used fresh. The complex of dithizone with Cd and Pb is red (Seregin and Ivanov, 1997).
Dimethylglyoxime $C_4H_8N_2O_2$ (M*r* = 116.1) (metallochrome indicator)	Ni	1% solution of dimethylglyoxime in 1.5% solution of NaOH in 0.05 M borax (Na2B4O7 · 10 H2O) (pH 9.8–10.4). Solution may be stored for a week. The complex of dimethylglyoxime with Ni is crimson (Seregin *et al.*, 2003).
Sodium rhodizonate $C_6Na_2O_6$ (M*r* = 214.0) (metallochrome indicator)	Sr	0.2% solution of sodium rhodizonate in distilled water. Solution may be stored for a week. The complex of sodium rhodizonate with Sr is grayish brown (Seregin and Kozhevnikova, 2004).
Zincon $C_{20}H_{15}N_4NaO_6S$ (M*r* = 462.4) (metallochrome indicator)	Zn	0.0065 g of zincon is dissolved in 0.2 ml of 1 M NaOH in borax (Na_2B_4O7 10 H_2O) solution (pH 9.8–10.4) and adjusted with distilled water to 10 ml so that the concentration of borax in the solution becomes 0.05 M; the obtained solution is heated to 80°C and cooled to room temperature. The solution may be stored for a week. The complex of zincon with Zn is blue (Seregin *et al.*, 2011).
Zinpyr_1 $C_{46}H_{36}C_{l2}N_6O_5$ (M*r* = 823.2) (fluorescent indicator). Excitation and emission maximums of Zinpyr_1 are located in the visible region: at 490 and 525 nm, respectively	Zn	In order to prepare 5 mM stock solution, a sample of reagent is dissolved in dimethyl sulfoxide. Stock solution is stored at –20°C in darkness. Directly before the analysis, reagent is thawed and diluted to 10 μM with super deionized water. In the presence of Zn, Zinpyr_1 produces green fluorescence (Sinclair *et al.*, 2007).

b- In detection of H_2O_2 and $O^{2\bullet-}$ a result of cadmium contamination

Romero-Puertas *et al.* (2004) studied the effect of cadmium on H_2O_2 and $O_2^{\cdot-}$ production in leaves from pea plants grown for 2 weeks with 50 μm Cd, by histochemistry with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively. The subcellular localization of the reactive oxygen species (ROS) was studied by using $CeCl_3$ and Mn/ DAB staining for H_2O_2 and $O_2^{\cdot-}$, respectively, followed by electron microscopy observation. In leaves from pea plants grown with 50 μm $CdCl_2$, a rise of six times in the H_2O_2 content took place in comparison to control plants. The accumulation of H_2O_2 was localized mainly in the plasma membrane, mesophyll and epidermal cells, as well as in the tonoplast of bundle sheath cells. In mesophyll cells, the accumulation of H_2O_2 was observed in mitochondria and peroxisomes. Localization of $O_2^{\cdot-}$ production was demonstrated in the tonoplast of bundle sheath cells, and plasma membrane from mesophyll cells. The Cd-induced production of the ROS, H_2O_2 and $O_2^{\cdot-}$, could be attributed to the phytotoxic effect of Cd. In this connection, lower levels of ROS were assumed to function as signal molecules for the induction of defense genes against the injurious effects of the heavy metal (Fig. 8).

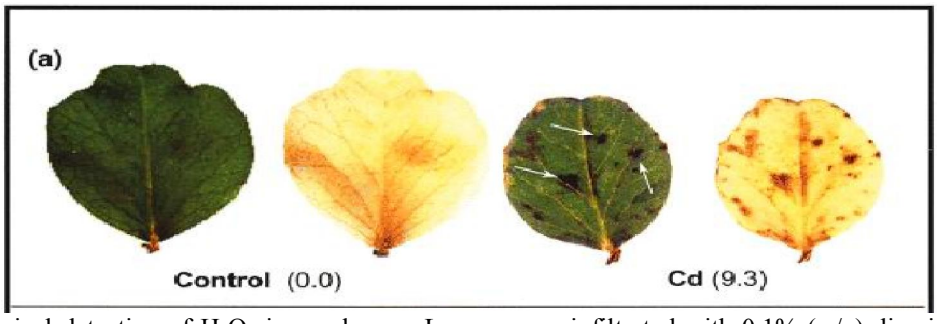


Fig. 8. Histochemical detection of H_2O_2 in pea leaves. Leaves were infiltrated with 0.1% (w/v) diaminobenzidine (DAB) for 8 h in the dark. (a) Leaves from control and Cd-treated plants. To visualize DAB deposits, leaves were decoloured in boiling ethanol (right leaves). Arrows indicate brown deposits of H_2O_2 (Romero-Puertas *et al.,* 2004).

However, Jin *et al*. (2008) found that exposure to cadmium resulted in significant ultrastructure changes in the root meristems and leaf mesophyll cells of *S. alfredii*. Damages were more pronounced in NHE even when Cd concentrations were one-tenth of those applied to HE. In the cadmium stress damaged chloroplasts resulted in imbalanced lamellae formation which is coupled with early leaf senescence. Histochemical results revealed that glutathione (GSH) biosynthesis inhibition led to overproduction of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2\cdot^-$) in HE but not in NHE. The GSH biosynthesis induction in root and shoot exposed to elevated Cd conditions, however, might be involved in Cd tolerance and hyper accumulation in HE of *S. alfredii* H (Fig. 9).

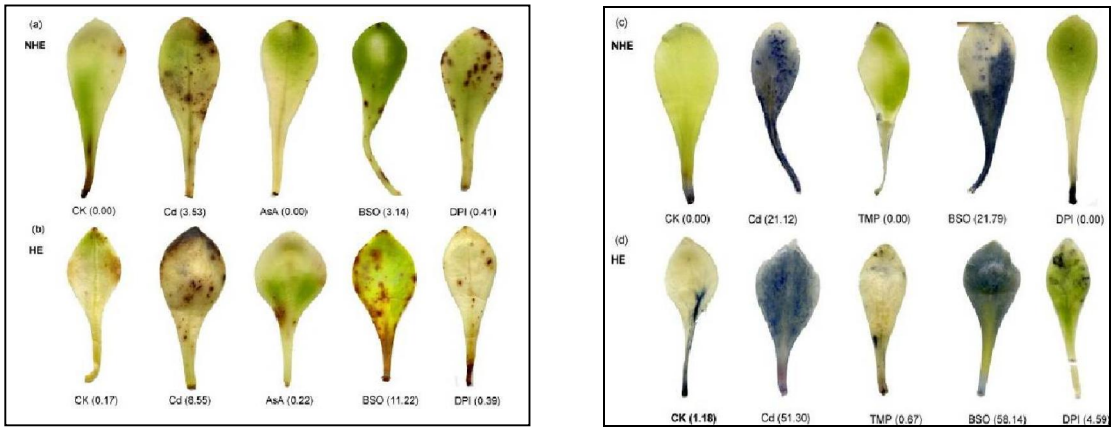


Fig. 9. Histochemical detection of H_2O_2 (a: NHE; b: HE) and $O^2\cdot^-$ (c: NHE; d: HE) in leaves of *Sedum alfredii* H. Excised leaves from both control and Cd-treated plants (NHE and HE) were grown in 10 μM and 100 μM Cd for 7d, respectively immersed in diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) solution to visualize brown or blue spots characteristic of DAB or NBT reaction with H_2O_2 and $O_2\cdot^-$, respectively. Then, leaves were bleached by immersing in boiling ethanol to visualize the spots.
Treatment symbols: AsA-H_2O_2 scavenger, TMP-$O_2\cdot^-$ scavenger, BSO- glutathione synthesis inhibitor, DPI-oxidase inhibitor. The results were expressed as percentage of spot area in pixels, vs. total leaf area [(spot area/ total leaf area) × 100]. Leaves were taken from control and Cd exposed plants, plus from Cd exposed plants treated with other compounds. The effect of AsA, TMP, BSO, and DPI on control plants is excluded; the percentage effect in treated plants is indicated in brackets. Results are reported from five representative individual experiments (Jin *et al*., 2008).

c-In the detection of aluminum accumulation, lipid peroxidation, callose production, and plasma membrane integrity

In peas (*Pisum sativum* L.) roots, staining were observed to distribute similarly on the entire surface of the root apex regarding aluminum accumulation, lipid peroxidation, and callose production). Meanwhile, the loss of plasma membrane integrity (detected by Evans blue uptake) was localized exclusively at the periphery of the cracks on the surface of root apex (Yamamoto *et al*., 2001).

They added that the enhancement of four phenomena, i.e. aluminum accumulation, lipid peroxidation, callose production and root elongation inhibition displayed similar aluminum dose dependencies which occurred at 4hs exposure. The loss of membrane integrity, however, was enhanced at lower aluminum concentrations and after a longer aluminum exposure of 8h. The addition of butylated hydroxyanisole (a lipophilic antioxidant) during aluminum treatment was found to completely prevent only the lipid peroxidation and callose production by 40%. Thus, lipid peroxidation was suggested to represent relatively early symptom induced by the accumulation of aluminum and appear to cause, in part, callose production. Whereas, the loss of plasma membrane integrity represented a relatively late symptom caused by cracks in the root due to the inhibition of root elongation (Figs. 10 & 11).

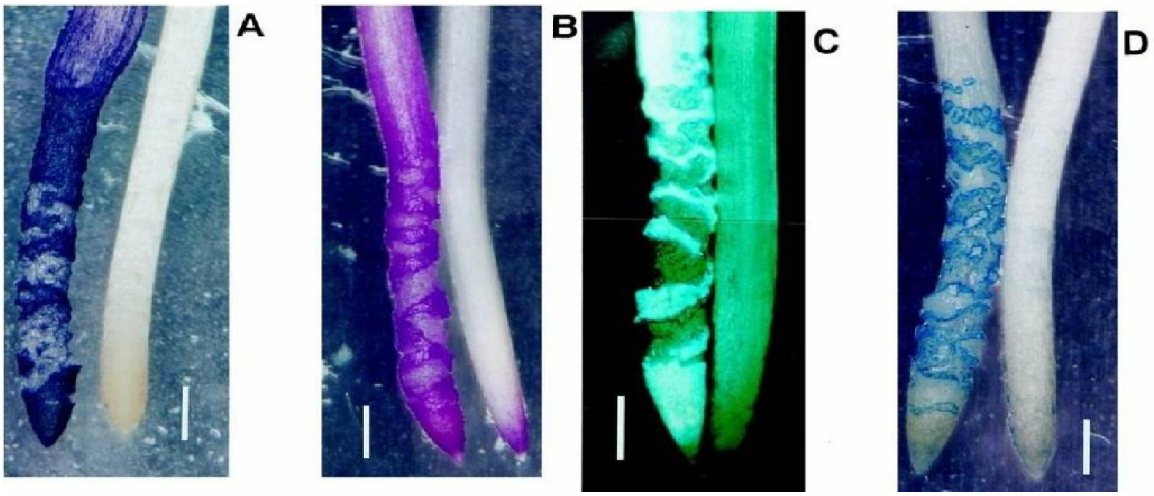


Fig. 10. Histochemical detection of lipid peroxidation and other events caused by aluminum in pea roots. Pea seedlings were treated with (left) or without (right) 10 µm aluminum in 100 µm $CaCl_2$ (pH 4.75) for 24h. The roots were stained with hematoxylin (A, aluminum accumulation), Schiff's reagent (B, lipid peroxidation), aniline blue (C, callose production), or Evans blue (D, the loss of plasma membrane integrity). The positive staining of each technique in the photomicrographs appears as bright images in panels A, B, and D and as a fluorescent image in panel C. The bar in each graph indicates 1 mm (Yamamoto *et al*., 2001).

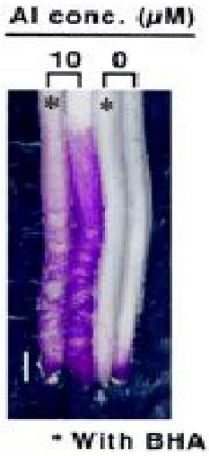


Fig. 11. Histochemical detection of Butylated hydroxyanisole (BHA) effect on the aluminum-enhanced lipid peroxidation in pea roots. Pea seedlings were treated with or without 10 µm aluminum in the presence (*) or absence of 20 µm BHA in 100 µmCaCl2 (pH 4.75) for 24 h. The roots were stained with Schiff's reagent for the detection of lipid peroxidation. The positive staining shows a bright image in the photomicrograph. Bar indicates 1 mm (Yamamoto *et al*., 2001).

5-In detection of active substances in aromatic plant; citral accumulation in lemongrass

Luthra *et al*. (2007) located the sites of citral accumulation in lemongrass (*Cymbopogon flexuosus* Nees ex Steud) wats (cultivar OD-19) by Schiff's reagent, which upon its reaction with aldehydes (citral) gives a purple-red colouration. Using this technique, single oil-accumulating cells were detected in the adaxial side of leaf mesophyll commonly adjacent to non-photosynthetic tissue and between vascular bundles. In this respect, however, the citral lacking cultivar GRL-1 (geraniol rich) leaf sections, which also was subjected to Schiff's reagent could be compared to the cultivar OD-19 leaf sections. In lemongrass mutant GRL-1, those specialized cells, however, are not be stained due to lack of citral. Hence, it could be confirmed that the observed schiff's staining reaction is associated with the accumulation of citral substance in a given cell (Fig. 12).

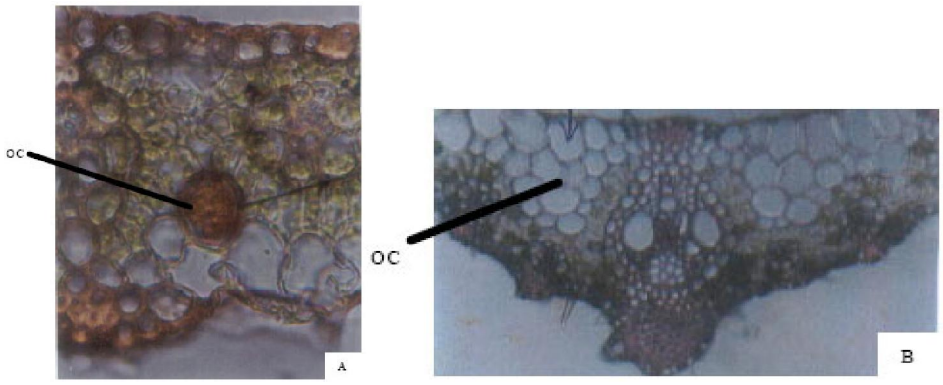


Fig. 12 (Luthra *et al*., 2007)
(A) Cross section of *Cymbopogon flexuosus* cv. OD-19 leaf showing the red colored oil cells (OC) as the citral accumulating site, (B) Cross section of mutant chemotype GRL-1 leaf showing colorless oil cells (OC) indicating the absence of citral

6. In localization of the site of monoterpene phenols` accumulation in plant secretory structures

Gersbach *et al.* (2001) reported a new histochemical method aimed to localize monoterpene phenols in essential oil secretory plant structures. The method was adapted from a spot test originally devised for the *in vitro* detection of phenolic compounds in organic analyses. Plants subjected to the test were the Lamiaceae species, i.e. *Thymus vulgaris* L., *Oreganum vulgare* L. and *Mentha x piperita* L. which are known to accumulate essential oils in their glandular trichomes.

A reagent consisting of 4-nitrosophenol in conc. H_2SO_4 was applied to leaf sample of each species. The positive reaction revealed the existence of phenol was indicated by the production of colored indophenols. Same method was employed in detection of monoterpene phenols in the trichomes of *T. vulgaris* (thymol) and *O. vulgare* (carvacrol), appeared as changes to red and green colors, respectively. Whereas, negative color reaction proved the absent of phenol in the trichomes of *M. x piperita*. The results were confirmed by GC-MS analysis of leaf volatile extracts from each species, and via an *in vitro* tests with thymol and carvacrol (Fig. 13).

It is suggested that this method could be used in a rapid field survey to identify the existence of bioactive compounds in certain plants.

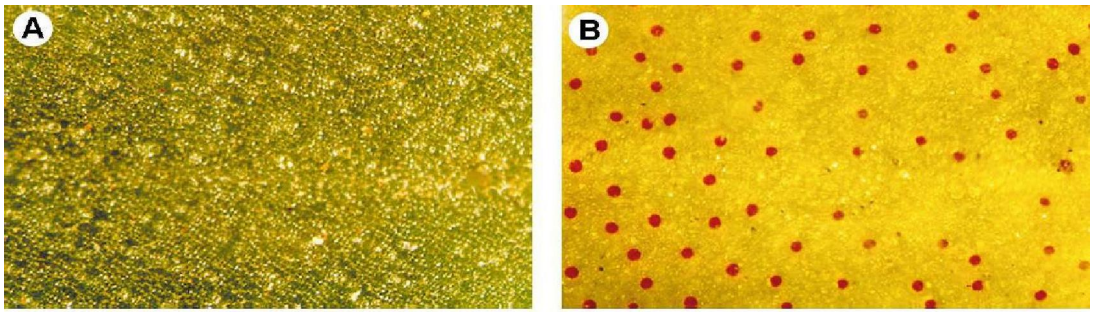


Fig. 13: A&B: Histochemical tests; A and B, Leaf of *Thymus vulgaris* before and after treatment with nitrosophenol reagent respectively; peltate glandular trichomes in B are stained red as a result of thymol condensing with nitrosophenol to produce a red indophenols (Gersbach *et al.* (2001).

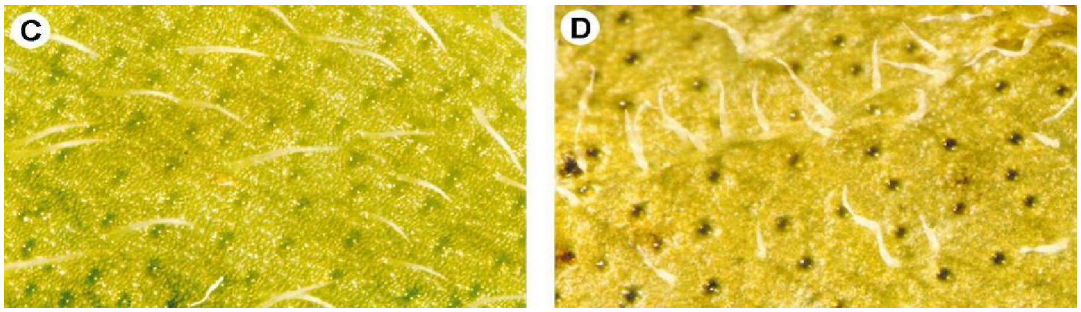


Fig.13: C & D. Leaf of *Oreganum vulgare* before and after treatment with nitrosophenol reagent respectively; peltate glandular trichomes in D are stained green as a result of carvacrol condensing with nitrosophenol to produce a green indophenols (Gersbach *et al.* (2001).

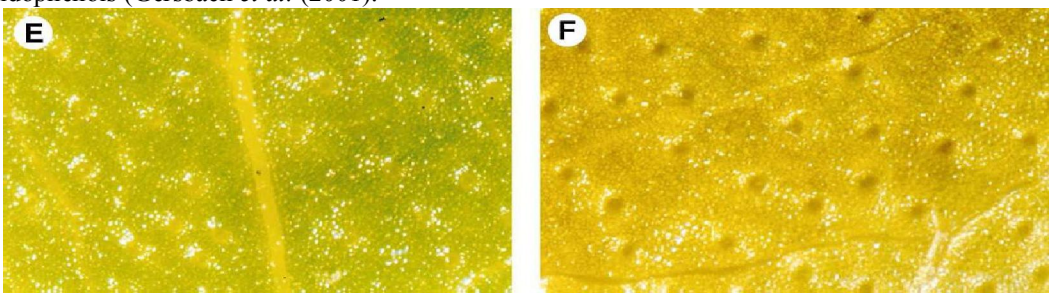


Fig. 13: E & F. Leaf of *Mentha piperita* before and after treatment with nitrosophenol reagent respectively; the glandular trichomes in F are not stained, indicating no reaction; therefore no phenol is present (Gersbach *et al.* (2001).

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