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Histochemical Localization of Citral Accumulating Cite in Lemongrass (*Cymbopogon flexuosus* Ness Ex. Steud) Wats Cultivar OD-19

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Abstract: The aerial parts (leaves) of lemongrass (*Cymbopogon flexuosus* Nees ex Steud) wats (cultivar OD-19) on steam-distillation yield an essential oil rich in acyclic monoterpenes, citral (83%). To specifically locate the sites of citral accumulation in lemongrass we employed Schiff's reagent, which upon 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 addition to cultivar OD-19, a citral lacking cultivar GRL-1 (geraniol rich) leaf sections, also stained with schiff's reagent and compared with that of lemongrass cultivar OD-19 leaf sections. In lemongrass mutant GRL-1, these specialized cells, however, could not be stained due to lack of citral. Hence, it is confirm that the observed schiff's staining is associated with accumulated citral.

Key words: Geraniol rich lemongrass (GRL-1), *Cymbopogon*, oil cells, geraniol, citral, schiff's reagent

INTRODUCTION

Histochemical and immunohistological methods have been developed for qualitative and quantitative analysis of virtually all cellular components, including proteins, carbohydrates, lipids, nucleic acids and the range of ionic elements in diverse non-secretory and secretory plant tissues (Gersbach *et al*., 2001; Bouvier *et al*., 2000). These methods in combination with various microscopic imaging techniques can be utilized in the study of essential oil secretion in plants. A number of species of *Cymbopogon*, such as palmarosa (*Cymbopogon martinii*), lemongrass (*C. flexuosus*), citronella (*C. winterianus*) and jamrosa (hybrid of *C. nardus* x *C. jwarancusa*) are the natural source of essential oils among others (Husain, 1994). *C. flexuosus* (cultivar OD-19), on steam distillation yields an essential oil rich in acyclic monoterpenes, citral (a and b), however, its mutant (cultivar GRL-1), lacks citral and contains geraniol, as the major component of essential oil. The citral and geraniol, is occupying ever-increasing and vastly varied significance owing to their specific recognition in the pharmaceutical, flavour and perfumery industries (Guenther, 1950). Monoterpenes are derived by condensation of two five-carbon units of isopentenyl diphosphate (IPP). IPP is produced either by the cytoplasmic classical acetate/MVA or plastidic non-mevalonate/methyl-erythritol phosphate or/Rohmer pathway (Lewinsohn *et al*., 1998; Rhomer *et al*., 1993; Lichtenthaler *et al*., 1997; McKaskill and Croteau, 1997; Luthra *et al*., 1999; Tholl *et al*., 2004). Monoterpene biosynthesis and accumulation is associated with the presence of specialized secretory structures, such as glandular trichomes, oil and resin ducts, or glandular epidermis, that compartmentalize these often toxic components from metabolically active cells (Fahn, 1988; Croteau, 1986). Previous studies in lemongrass, has shown the presence of oil drops in adaxial epidermal and mesophyll cells, as well as in bulliform and phloem cells (Tsai, 1978; Ming *et al*., 1996). In *C. winterianus,* five types of glandular microhairs, on the abaxial surface of epidermis have found to accumulate essential oil. Also, their numbers are correlated with the essential oil content and composition (Iruthayathas and Herath, 1982). A citral accumulating single cell have been identified, histochemically in *C. citratus* by the aldehyde specific Schiff's reagent (Lewinsohn *et al*., 1998). Monoterpene phenol accumulation in the trichomes of *Thymus vulgaris* L., *Oraganum vulgare* L. and *Mentha x piperita* L. have recently been identified using a reagent consisting of nitrosophenol in concentrated H_2SO_4 (Gersbach *et al*., 2001). Here, we report histochemical

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localization of citral (monoterpene aldehyde) by aldehyde specific Schiff's reagent in *C. flexosus* (cultivar OD-19). In addition to cultivar OD-19, citral lacking mutant (cultivar GRL-1) have also been stained to further confirm that the observed schiff's staining is associated with accumulated citral. Our studies have shown the presence of oil accumulating cells in the adaxial side of leaf mesophyll, commonly adjacent to non-phtotosynthetic tissues and between vascular bundles. However, in mutant (cultivar GRL-1), these specialized cells, however, could not be stained.

MATERIALS AND METHODS

Plant material: *Cymbopogon flexuosus* Ness ex Steud) wtas (cultivars OD-19 and GRl-1 (mutant) plants were raised from slips at the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow by following standard agronomic practices.

Tissue preparation and staining: Tissue preparation and staining was performed according to Lewinsohn *et al.* (1998). Hand cut sections were prepared from fresh and young leaves and incubated in Schiff's reagent for 30 min at room temperature. The sections were than washed thrice for 10 min each with freshly prepared solution of 0.5% (w/v) sodium metabisulphite in 0.1% HCL. Stained sections were than examined under a light microscope Optiphot pol (Nicon) at 10×10×1000 magnifications (O'Brien and McCully, 1981). Schiff's reagent was prepared by dissolving 1% (w/v) pararosaniline chloride and 4% sodium bisulphate in 0.25 N HCL.

Determination of essential oil composition: The essential composition was determined after hydro-distillation of fresh leaves (100 g) in Clevenger apparatus using gas liquid chromatography. The GLC analysis for major oil constituents was performed using a Perkin Elmer 3920 B apparatus equipped with FID, stainless steel column (2 m×3 mm) packed with 10% FFAP (free fatty acid phase) on chromosorb WAW (80-100 mesh). The operating conditions were as follows: column temperature 200 and 250°C, respectively. Nitrogen was used as a carrier gas and its flow rate was adjusted to 30 mL min^{-1}. The citral, geraniol and geranyl acetate peaks were identified by co-injecting authentic standards and quantified using a Varian integrator (model 4400). Minor constituents were analyzed using Perkin Elmer 8500 gas chromatograph equipped with FID using BP-1 (diethyl polysiloxane) column (30 m×0.32 mm i.d. and 0.25 micron film thickness and nitrogen was used as carrier gas at 7 psi inlet pressure. Temperature programming was performed from 60 to 220°C at 5°C min^{-1} and the split ratio was 1:80. The identification of various oil constituents was done by comparison of their Kovats retention indices on BP-1 column (relative to C*-C23 alkanes) with their literature values and peak enrichment on co-injection with authentic samples wherever possible. The peak area percentage was calculated on BP-1 column without the use of correction factors.

RESULTS AND DISCUSSION

The essential oil samples obtained after hydro-distillation were analyzed for their chemical composition by gas chromatography (GC). 28 compounds in the oil from cv. OD-19 (oil yield 1.51%; %V/DW) and 20 compounds in its mutant cv. GRL-1 (oil yield 1.6%; %V/DW) were identified. Geranial (49.20%) and neral (33.42%) were identified as the major constituents in the oil from cv OD-19 with other constituents (>1.0%) borneol (2.23%), isopulegol (1.58%) and 6-methyl hept 5-en-2one (1.21%). Geraniol accounted for 89.39% of the total oil with other constituents (>1.0%) geranyl acetate (3.83%) and y-terpinene (1.02%) in cultivar GRL-1 (mutant). Rest of the constituents, were present in minor <1.0%) amounts in both the cultivars.

The method used here to detect monoterpene aldehydes is the example of chemical tests that have been adapted for histochemical localization of citral (essential oil). Because such methods are based on the specificity of a reaction between a reagent and the functional group of a compound, potential subjects would be plant species that accumulate significant amount of a monoterpene having a particular functional group. Essential oils like other natural products, have found to accumulate in the epidermal oil glands (Croteau and Johnson, 1984; Gang *et al.*, 2001). The presence of secretory structures appears to be a prerequisite for monoterpenoid synthesis (Croteau and Johnson, 1984). However, the link between the synthesis and accumulation of essential oil and presence of secretory structures does not hold true for the subgenus *Strutia* of *Gossypium* (Brubaker *et al.*, 1996). In lemongrass cultivar OD-19, the major components in essential oil are the aldehydes geranial and neral, an aldehyde staining method was employed to histochemically locate the sites of citral accumulation. The cross sections of lemongrass cultivar OD-19 leaf blades, when incubated with Schiff's reagent, only discrete single cell stained with Schiff's reagent. These oil cells were

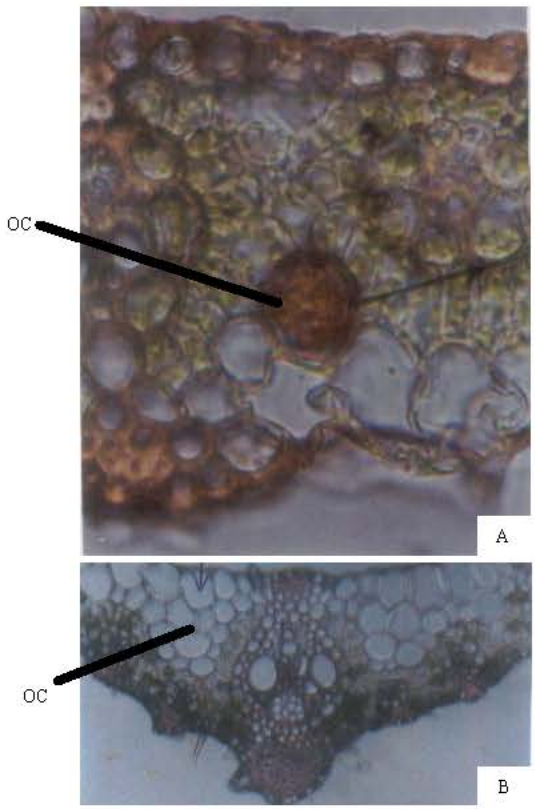

Fig. 1: (A) Cross section of *Cymbopogon flexuosus* cv. OD-19 leaf showing the red colored oil cells (OC) as the citral accumulating site (B) Cross section of mutant chemotype GRL-1 leaf showing colorless oil cells (OC) indicating the absence of citral

found to possess a dense content and gave an intense red-purple coloration upon reaction with schiff's reagent, indicating the presence of aldehydes, geranial and neral. The oil cells are parenchymatous cells embedded in the adaxial side of the leaf mesophyll commonly adjacent to non-photosynthetic tissues, between the vascular bundles (Fig. 1A). The oil cells are morphologically indistinguishable from their neighbouring parenchymal cells, except that they contain secreted material. Study with cultivar GRL-1 (mutant) leaf section has confirmed that the observed schiff's staining is associated with accumulated citral in cultivar OD-19, as in cultivar GRL-1 (mutant), the oil cells could not be stained with Schiff's reagent due to lack of aldehydes (citral) (Fig. 1B). The results are consistent with that of previous studies concerning the presence of oil glands (cells) in *C. winterianus* and *C. citratus* (Lewinsohn *et al*., 1998) where the citral is accumulated in single cell on the adaxial side of leaf mesophyll and between vascular bundles. Schiff's reagent reacts with aldehydes to form Schiff's bases, often containing conjugated double bond systems that produce intense and typical colorations (O'Brien and McCully, 1981). Schiff's reagent is water-based and therefore, displacement of lipophilic materials such as citral during staining was not observed. Thus, the histochemical localization of citral accumulating sites in lemongrass using Schiff's reagent is more reliable than ethanol-based lipid stains. However, a polygodial, a sesquiterpene aldehyde present in *Polygonum hydropiper* does not seem to produce a colour when reacted with Schiff's reagent (Lewinsohn *et al*., 1998).

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