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Foliar chemical attributes of the hybrid bred from *Eucalyptus citriodora* x *E. torelliana* and its parental taxa, and implications for fungal resistance

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Abstract. One of the important aspects of hybridization is to understand the interaction between hybrid plants and the pests and diseases of the parental taxa. The foliar chemical attributes were compared between the hybrid of *Eucalyptus citriodora* and *E. torelliana* and its parental taxa. The fungus, *Cylindrocladium quinqueseptatum*, to which the hybrid and one parent *E. torelliana* have been observed resistant in the field, was used to examine patterns of resistance in relation to foliar constituents found active in laboratory bioassays. Concentration of active constituents of the hybrid was higher (monoterpenes- α-pinene, β-pinene and citronellal, and total phenolics) than either parent or equivalent (ursolic acid) to parent *E. torelliana* thus suggesting an resistance pattern of hybrid. β-pinene, ursolic acid and total phenolics were found to be heritable. The findings suggest a chemical basis for fungal resistance and also indicate that the constituents could be used for screening of the disease resistant progeny in this tree system.
Keywords *Eucalyptus citriodora* and *E. torelliana*, *Cylindrocladium quinqueseptatum*, foliar chemical attributes, ursolic acid, monoterpenes, phenolics.

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Introduction

Hybridization occurs in every major plant taxon (Floate & Whitham 1994) and artificial hybridization is a common procedure in agriculture and silviculture in light of the fact

that hybrid can combine desirable features of parental types or display novel phenotypes as a result of changes in genetic composition (Strauss 1994). An important aspect of hybridization is the interaction between hybrid plants and the pests and diseases of the parental taxa. The comparison of pest and disease preference and performance on parental plants with that on hybrids can provide insights into the inheritance of potential resistance mechanisms (O' Reilly Wapstra et al. 2005), and host shift mechanisms that may in turn explain the distribution of insect species among plants (Thompson 1988).

Eucalyptus, in the family Myrtaceae, is a genus of over 800 species. *Eucalyptus* species are of commercial importance and used predominantly for pulp and paper (Tewari 1992). Hybrid breeding of eucalyptus for forest plantations is a silvicultural strategy adopted in many eucalypt – growing reasons worldwide, to maximize tree performance by combining the desirable traits of different species (Assis De 2000). Eucalyptus are known to form hybrids readily with related species (Griffin et al. 1988**)**. Traits for improvement through hybridization include growth rate, ability to coppice and propagate, pulp yield, wood density, resistance to frost, drought and salinity (Dale & Dieters 2007) and pests and diseases (Potts & Dungey 2004). One prominent characteristics of the group is the high essential oil content of the leaves, and the oils vary substantially among taxa (Bingell et al.1998, Dunlop et al. 1999, Asante et al. 2001, Keszei et al. 2008), thus affecting feeding preferences of insect herbivores (Edwards et al. 1993, Steinbauer et al. 2004). Other leaf characteristics - including waxes (Edwards 1982), phenolics (Landsberg 1990, Andrew et al. 2005), nitrogen content (Ohmart & Edwards 1991), and physical attributes such as leaf toughness, moisture content, specific leaf weight, lamina thickness and leaf surface galbrousness - have also been reported to directly affect eucalypt susceptibility to insect attack (Edwards & Wanjura 1990, Nahrung et al. 2009). An understanding of how characters important to plant herbivores (for instance, secondary chemicals and physical leaf characteristics) vary between species and their hybrids contributes to understanding of the mechanisms of host choice by insect herbivores and selection of resistance to the insect pests (Nahrung et al. 2009, Hallgren et al. 2003).

Cylindrocladium quinqueseptatum (CQ), the most destructive pathogen of *Eucalyptus,* is wide spread and occurs on eucalyptus seedlings in nurseries, plantations or in small trial plots. This fungus causes *Cylindrocladium* leaf and seedling blight (CLSB) disease and is most often fatal. A hybrid of *E. citriodora* (EC) and *E. torelliana* (ET) bred at Forest Research Institute, Dehra Dun has significant advantages in biomass accumulation. The hybrid and one of its parents ET have been observed resistant to the CLSB in the field (Tewari 1992). This resistance, was however, subjective and it was hypothesized that the foliar resistance of the hybrid to CQ may be derived from foliar chemical constituents, hence our objective was to characterize chemical constituents conferring resistance to the foliage against CQ, for the development of methods for early selection and evaluation of progeny performance, using these marker constituents in this tree system. Therefore, we examined laboratory antifungal assay directed foliar chemical characteristics of the hybrid (EC x ET) and its parental taxa (EC and ET) and variations of the active constituents in each of the taxon.

Materials and methods

Plant material

The foliage used in the study were matured ones from the trees of the three taxa planted in experimental plots of the Genetics Division of the institute. Replicate samples (N = 5) of foliage from 5 randomly selected plants of each

taxon were collected, cut into small pieces and processed as follows.

Isolation of essential oils

Fresh leaves (100 g) were hydrodistilled using Clevenger apparatus for a period of three to four hours. The aqueous distillate so obtained was treated with diethyl ether and the etheral portion was dehydrated using anhydrous Na_2SO_4. Removal of the solvent on a gently heated water bath yielded colorless essential oils. All the essential oils were stored at 4°C until bioassayed and analyzed. The yield percentaje of the e-ssential oils isolated from EC, ET and EC x ET was 1.2, 0.03 and 0.03, respectively.

Characterization of monoterpenes in essential oils

The oil samples were diluted with the hexane (1:1000) and analyzed on a Hewlett Packard 5890 Series II gas chromatograph (GC) and Hewlett Packard 5971 Series Mass Selective Detector. Column used was a J&W Scientific Durabond – 5MS column (30 m x 0.25 mm x 0.25 µm). The GC conditions used were initial temperature 50°C followed by a rate of 5°C min^{-1} up to a final temperature of 210°C (6 min), carrier gas helium at constant flow 1.5 mL min^{-1}, split ratio 1:100, transfer line temperature 250°C. The MS was held at 250°C in the ion source with one scan per minute acquired. The monoterpenes were characterized by comparing retention times of the peaks with those of commercial standards (*α*-pinene, *β*-pinene, *p*-cymene, 1,8-cineole, citronellal and limonene, source Sigma Aldrich) and the National Institute of Standards and Technology (NIST) mass spectral library. The concentration of each of the identified monoterpenes was determined using the calibration curves drawn for the standards.

Isolation of extractives

The shade dried leaves of EC x ET were powdered and extracted sequentially with petroleum ether (60-80°C), acetone and 70% aqueous methanol using soxhlet extraction and respective extracts (LPE, LACE, LMET) were isolated after removal of the solvent under vacuum. The aqueous methanol extract was concentrated in vacuum and fractionated with ethyl acetate and *n*-butanol sequentially to yield ethylacetate (LMETEA) and *n*-butanol (LMETBU) fractions, respectively. Solvents from these fractions were removed under vacuum and respective extracts were obtained. All these extracts were stored at 4°C until bioassayed and analyzed.

Isolation of ursolic acid

The LACE was concentrated to dryness to determine the yield. But while repeating the experiments the acetone extract was concentrated to 200 ml and allowed to stand overnight. A solid got separated which was filtered and washed with acetone to obtain a white solid (5.35 g). Column chromatography of the solid over silica gel (100-200 mesh) using gradient elution with chloroform–methanol yielded one pure compound which was characterized as ursolic acid by direct comparison with authentic sample.

Quantification of ursolic acid by HPTLC

The extract used was obtained by extracting shade dried foliage with petroleum ether: acetone (4:1) for 4 hours followed by removal of the solvent in vacuum. HPTLC analysis was carried out on a CAMAG HPTLC system. A working standard solution (concentration 67%) of ursolic acid (9 mg) in methanol (10 mL) was prepared and applied (2-10 µl) with samples (EC 2, 3, 4 µL / ET 2, 3, 4, 6 µL /EC x ET 4, 5, 6 µL to the silica gel TLC plate (60 F 254, Merck, Germany, 20 × 10 cm) as 6 mm

bands with automatic TLC applicator Linomat 5 equipped with 100 µL syringe with N_2 flow, 8 mm from the bottom. The plates were developed to a height of 8 cm in a twin trough glass tank using 25% ethyl acetate: hexane with presaturation for 20 minutes. After removal from the chamber, the plates were completely dried in air at ambient temperature, derivatized with 10% methanolic H_2SO_4 followed by heating at 120°C for 2 minutes, scanned and quantified at 515 nm using Scanner 3 with Wincats 3.2.1 software. All the experiments were performed in triplicate and the results were averaged.

For calibration different amounts (2-10 µL) of standard ursolic acid equivalent to eight different concentrations (240-1200 ng/ band) with double spotting of 3.5 µL were applied in triplicate on three plates separately and chromatographed as described above. The regression coefficient (R^2) and % residual standard deviation (RSD) were determined.

Determination of total phenolics contents (TPCs)

The powdered leaves were defatted with n-hexane and defatted samples (5 g each) were extracted with 50% acetone water (50 ml) at ambient temperature for 24 hours. The extracts were filtered and stored at 4°C until analyzed further. TPC was estimated using the Folin – Ciocalteau colorimetric method and the results were expressed as g gallic acid equivalents (GAE) / 100 g of the leaves.

Laboratory antifungal assays

The fungal strain, CQ used in the study was obtained from the culture library of the institute. The antifungal assay was performed using poison food technique. The extracts / fractions and pure compound (ursolic acid) were dissolved in minimum amount of ethanol and methanol, respectively. Along with the test chemicals, control plates were also inoculated and incubated in dark at 27°C and 70% relative humidity. Each of the four treatments consisting of control and different concentrations of the test chemicals were replicated three times. When the mycelium of fungus reached the edges of the control dishes, the lowest concentration with no sign of growth was defined as minimum inhibitory concentration (MIC) as a measure of antifungal activity.

All chemicals and reagents were of laboratory / analytical grade (for quantitative analysis) and purchased from standard commercial suppliers. GENSTAT Version 5 was used to carry out the determination of heritability (% Broad sense)

Results

The composition of monoterpenes identified in foliage of each taxon is given in Table 1. The relative amount of the compounds was varied among taxa. The monoterpenes identified were common component of eucalypt leaf chemistry (Keszei et al. 2008). *α*-pinene, *β*-pinene, *p*-cymene, citronellal and limonene detected in hybrid were also present in EC

Table 1 The composition of the monoterpenes in essential oils of leaves of EC, ET and EC x ET

Monoterpene	RT (in minutes)	EC	EC x ET	ET
		Mean precentage area		
α-pinene	6.3	0.14	0.77	0.14
β-pinene	9.3	0.22	0.41	0.06
p-cymene	10.8	0.16	0.19	Not detected
1,8-cineole	11.0	1.85	Not detected	Not detected
Limonene	13.9	0.32	0.41	0.04
Citronellal	14.9	74.65	0.45	0.24

while *p*-cymene was not detected in ET. 1,8-cineole present in the EC was absent in the hybrid and ET. *α*-pinene, *β*-pinene, *p*-cymene and limonene present in hybrid recorded higher concentration than either parent. The concentration of citronellal (0.45%) in the hybrid was much less than that in EC (74.65%) and little more than that in ET (0.24%). The individual monoterpenes common to each taxon were subjected to the laboratory bioassay against the CQ of which *α*-pinene, *β*-pinene and citronellal demonstrated bioactivity (Table 2).

Soxhlet extraction of the hybrid foliage using petroleum ether (b.p. 60-80°C), acetone and 70% aqueous methanol afforded their respective extracts (LPE, LACE and LMET). TLC examination of LPE showed the presence of chlorophyll and fatty compounds and was not examined further. The extracts LACE and LME were laboratory bioassayed against the CQ. Both the extracts showed antifungal activity (Table 2). One pure compound, ursolic acid (UA), the major component, was isolated from column chromatography of ACE. The UA when bioassayed against the CQ was also found active (Table 2).

The concentration of UA in the foliage of each taxon was determined by HPTLC method. Development of silica gel TLC plate (60 F 254) in a twin trough chamber for 20 minutes with 25% ethylacetate: hexane as mobile phase resulted in good separation of UA. The compound with R_F value 0.22 was identified as ursolic acid. Regression analysis of linearity data for UA showed that the response was linearly dependent on amount of the UA in the range of 240-1200 ng with regression coefficient. The spots for UA in the extracts were confirmed by comparing the R_F values with those for the pure UA. Spectral studies revealed that the peaks obtained from the extracts and the standard were identical because of their similar spectral pattern. The values of R^2 and % RSD (Table 3) determined through the regression analysis of data obtained in the assay experiment carried out with the UA standard and the extracts indicated that the precision of the method was reasonably good. From the above data the content of UA were determined and found to be close to either parent (Table 4).

The LMET was fractionated with ethyl acetate and n-butanol sequentially to afford respective fractions - LMETEA and LMETBU. These fractions were also bioassayed against the CQ. The LMETEA demonstrated bioactivity (Table 2) while LMETBU remained inactive. HPTLC examination (mobile phase: ethylacetate formic acid: acetic acid: water, 100:11:11:27) of LMETEA revealed the presence of flavonoids which on the basis of color reaction with 1% Vanillin H_2SO_4, UV λ_{max} and RF values were found to be catechin / epicatechin, flavonols and flavanols (Table 5). Several polyphenolic compounds such as flavonoids (flavones, flavanones, dihydroflavonols, flavonols and flavanols) and phenolics acids (Conde et al. 1997,

Table 2 MIC values of compounds and extracts found active in the laboratory antifungal assay against CQ

S.No.	Compound / Extract	MIC (%)
	Compound	
1	*α*-pinene	0.50
2	*β*-pinene	0.25
3	Citronellal	0.13
	Extract	
4	LACE	1.00
5	LME	0.12
6	UA	1.00
7	LMETEA	0.12

Table 3 Regression analysis data of HPTLC assay experiment carried out with the UA standard and the extracts of the foliage of EC, ET and EC x ET

Taxon	R^2	% RSD
EC	0.99928	1.10
ET	0.99948	0.59
Ec x ET	0.99923	1.07

Table 4 Concentration of UA and total phenolics contents (TPCs) in the foliage of of EC, ET and EC x ET

Taxon	UA (%)	TPC s (g GAE / 100 g of the leaves)
EC	0.99	0.51
ET	1.07	0.51
EC x ET	1.09	0.57

Table 5 TLC characteristics of the flavonoids detected in the extract LMETEA

Flavonoid type	Color	$UV_{\lambda max}$	R_F
Catechin / epicatechin	Red	228 (sh), 279	0.95
Flavonols	Yellow-orange	266,353	0.74
		269,367	0.64
		273,365	0.51
Flavanols	Red	281	0.38
		276	0.29

Horn et al. 1964, Lamberton 1964, Hillis & Isoi 1965, Wollenweber & Kohorst 1981) have been reported in leaves of different *Eucalyptus* species. Total phenolics contents (TPCs) in foliage of each taxon were, therefore, determined and compared. TPCs in the foliage of hybrid were higher than either parent (Table 4).

Heritability (% broadsense) of the three bioactive monoterpenes, UA and the total phenolics was estimated. Amongst the monoterpenes, only *β*-pinene was highly heritable (H 90.6%) while α-pinene and citronellal were not heritable. Heritability of UA was found to be relatively low (H 37.06%) whereas total phenolics demonstrated high heritability (H 93.98%).

Discussion

Studies of susceptibility of plant species and the hybrids to pests and diseases have been done considerably. Hybrid susceptibility (arising either through dominance to a susceptible parent, or a hybrid that is more susceptible than either parent) appears the most common pattern while hybrid resistance (arising either through dominance to a resistant parent, or a hybrid that is more resistant than either parent) appears to be reasonably rare while in some of the studies an additive pattern, whereby hybrid traits are intermediate between the two parental types, and almost no difference between parents and hybrids has been found (Fritz et al. 1999, Dungey & Potts 2003, Hallgren et al. 2003, O' Reilly Wapstra et al. 2005). The hybrid (EC x ET) exhibited traits superior to the parent species for the foliar chemical characteristics investigated. The concentration of the foliar constituents (monoterpenes *α*-pinene, *β*-pinene and citronellal, UA, and total phenolics) conferring resistance to fungi, CQ in laboratory bioassays were higher (monoterpenes and total phenolics) in the hybrid than either parent or equivalent (UA) to parent ET. Monoterpenes have found applications in forest genetics as biochemical markers in in chemotaxonomy and in selecting less susceptible chemotypes to pests and diseases (Baradat et al. 1991, Hanover 1992, Michelozzi et al. 1995, Michelozzi 1999). Within eucalyptus, terpenes have been implicated in many ecological interactions including resistance to pests and diseases (Morrow & Fox 1980, Lawler et al. 1999, Eyles et al. 2003, Alves et al. 2004). UA, a triterpene occurring in concentration upto 2.5% in the *Eucalyptus* foliage has been reported to possess an array of biological activities including antifungal activity (Shukla et al. 1992, Dayal 1982). Although hybrid susceptiblity to herbivores is predicted in *Eucalyptus* (Dungey & Potts 2003, Potts & Dungey 2004), the hybrid taxon displayed resistance pattern in our study. Our findings also suggest a possible chemical basis for the hybrid resistance to CQ. Heritability estimates of the active constituents also show and that use of the contents of *β*-pinene, ursolic acid and total phenolics is possible for screening of CLSB resistant progeny in EC x ET system. This is the first report on chemical resistance of the hybrid bred from EC and ET to the fungus, CQ.

Conclusion

Cylindrocladium quinqueseptatum (CQ), the most destructive pathogen of *Eucalyptus,* causes *Cylindrocladium* leaf and seedling blight (CLSB) disease on *Eucalyptus* seedlings in nurseries, plantations or in small trial plots. A hybrid (EC x ET) bred from *E. citriodora* (EC) and *E. torelliana* (ET) has significant advan-

tages in biomass accumulation. The hybrid and one of its parents ET have been observed resistant to the CLSB in the field. This resistance was, however, subjective and it was hypothesized that the foliar resistance of the hybrid to CQ may be derived from the foliar chemical constituents. Laboratory antifungal assay directed foliar chemical characteristics of the hybrid (EC x ET) and its parental taxa (EC and ET) and variations of the active constituents in each of the taxon were studied. Three monoterpenes (α-pinene, β-pinene and citronellal), ursolic acid, and total phenolics conferring resistance to fungi, CQ were identified. Concentration of these active constituents of the hybrid was higher (monoterpenes α-pinene, β-pinene and citronellal, and total phenolics) than either parent or equivalent (ursolic acid) to parent ET thus suggesting an resistance pattern of hybrid. β-pinene, ursolic acid and total phenolics were found to be heritable. The findings suggested a possible chemical basis for the hybrid resistance to CQ and that use of the contents of β-pinene, ursolic acid and total phenolics is possible for screening of CLSB resistant progeny in EC x ET system.

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