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CHEMICAL CONSTITUENTS IN HDBRG TOBACCO*

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***Harvel De Bouxo Rio Grande* (HDBRG) tobacco is grown in the Guntur tract of Andhra Pradesh and is used in cigarette blends. The report that HDBRG tobacco is a rich source of solanesol has prompted to take up detailed investigations on the chemical quality parameters, lipophilic constituents and neutral volatile compounds responsible for flavour. HDBRG was found to be high in nicotine (2.9- 4.9%); low in reducing sugars (0.5 – 0.9%) and chlorides (0.2 – 0.3%). In respect of nutrient composition, the total nitrogen was high (2.9 – 3.8%), potassium was medium (0.7 – 1.7%) and phosphorus was low (~ 0.2%). Solanesol content in the samples varied from 1.60 to 2.80% with mean values of 1.97 and 2.46% for the seasons 2009-10 and 2010-11, respectively. Total hexane solubles or petroleum ether extractives (PEE) content in HDBRG tobacco was high (~ 10%) and solanesol accounted for ~ 25% of the extract. Further investigations were initiated for characterization of the non-polar and polar fractions which account for 5.8 – 8.3% and 1.1 – 2.1% of the hexane extract, respectively employing techniques like GC-MS and LC-MS. In the neutral volatile fraction, neophytadiene, megastigmatrienone isomers, duvatrienediol, thunbergol, dibutylphthalate, solanone, (E,E)-farnesylacetone, 4-oxo-beta-isodamascol and phenylacetaldehyde accounted for ~96% of the compounds. Nerolidol (0.7%), rishitin (0.7%), neryl acetone (0. 5%), (12z)-abienol (0.3%), phenylethyl alcohol (0.3%), indole (0.2%), viridiflorol (0.2%), 3-hydroxysolavetivone (0.1%), â-damascone (0.03%) and 14-labdien-8-ol (0.05%) are the other important flavour compounds identified. It is inferred from the data that presence of higher levels of these compounds could be attributed to higher nitrogen and nicotine contents in HDBRG leaf thus indicating their positive impact on the smoke flavour.**

INTRODUCTION

Harvel De Bouxo Rio Grande (HDBRG) tobacco, popularly known as HD burley tobacco, which is a high yielding, sun-cured, burley type of tobacco supposed to be introduced from Brazil. The heavy bodied HDBRG tobacco, which is closely related to burley in chemistry, grown under irrigated conditions in the heavy black soils of Chilakaluripet, Thakkellapadu and Pasumarru areas of Guntur district to the extent of 4000 ha with an annual production of 8 million kg of leaf. The crop is exclusively grown on conserved soil moisture during *Rabi* season extending from October - November to February - March. Deep black clay loams with neutral to slightly alkaline reaction are suitable for HDBRG tobacco cultivation. These black soils are characterized by low nitrogen, low organic carbon, low to medium phosphorus and high potassium status. The crop responds well to nitrogenous and phosphatic fertilizers (N:P:K :: 150:50:50 kg/ha). This leaf is mainly used in the domestic cigarette blends and only a small quantity is exported. This has characteristic flavour and aroma which varies with the type of irrigation and water used. It is a high nitrogen and high nicotine crop with low sugars and has a characteristic Burley nose with a reasonably good filling power. The nicotine levels normally range from 2.5 to 5.0% (Krishnamurthy and Deo Sigh, 2005).

Phani Kiran *et al.* (2008) reported that HDBRG tobacco could be a rich source of solanesol (Range: 0.50 – 3.75%; Mean: 1.75%). This finding has paved the way for detailed investigations on the chemistry of this tobacco type. Information on leaf quality parameters, nutrient composition and preliminary information on lipophilic constituents & neutral volatile compounds responsible for flavour are presented in this paper.

MATERIALS AND METHODS

Sample collection

Nine HDBRG tobacco leaf (~1 kg) samples each from the mid-stalk position were collected

during 2009-10 and 2010-11 seasons from bulk crop grown at CTRI Research Station, Guntur, Andhra Pradesh. Mid-ribs were removed from the leaf and the lamina portion was dried at 60 °C in an oven. The dried leaf lamina was powdered in a Wiley mill to pass through a 0.1 mm sieve for the analysis of different chemical constituents *viz.*, nicotine, reducing sugars, chlorides, solanesol, total nitrogen, phosphorous, potassium, neutral volatile compounds and petroleum ether extractives (PEE)/hexane extractives.

Analysis of leaf constituents

Standard methods were adopted for the determination of nicotine and reducing sugars (Harvey *et al.*, 1969); chlorides (Hanumantha Rao *et al.*, 1980); solanesol (Narasimha Rao *et al.,* 2000); total nitrogen (TIM, 1977); phosphorous and potassium (Jackson, 1973) employing auto-analyser (Model AA3, Bran + Luebbe, Germany), HPLC (Model, LC 8A, Shimadzu, Japan), auto-analyser (Model AA2, Bran + Luebbe, Germany), PC Based Double Beam Spectrophotometer (Model, 2202 Systronics, India) and flame photometer (Model, 128 Systronics, India), respectively.

Petroleum ether extractives (PEE)/Hexane extractives

Tobacco powder (500 g) was placed in a Whatman No. 1 extraction thimble, covered with a small plug of fat free cotton and extracted with approximately 3 l of n-Hexane (B.P : 64-70 °C) in a Soxhlet extraction apparatus fitted with a 5 l round-bottom glass joint flask. The extraction process was continued for 8 h, the solvent was removed using a Buchi flash evaporator at 40 °C, dried for 1 h in convection - type oven at ~60 °C, cooled in desiccator, weight of total extractives was recorded and per cent of total hexane extractives was calculated. The extraction was carried out in triplicate with the samples of the two seasons and mean values were calculated.

Fractionation of lipids

The total hexane extractives (triplicates of both the seasons) thus obtained were taken in 2 l separating funnel, dissolved in 600 ml of n-hexane, the hexane solubles were successively extracted four times (3 x 250 ml and 1 x 100 ml) with 90% methyl alcohol and the pooled extract was collected separately. The hexane layer was washed three times successively with a total of 450 ml of distilled water and the water solubles were discarded. Methanol and hexane extracts were concentrated by a Buchi flash evaporator at 40 °C, dried in desiccator, weights were recorded and per cent of non-polar and polar fractions was calculated.

Neutral volatile compounds

Tobacco powder (10 g) and 5 g of sodium sulphate were taken in 500 ml distillation flask, 250 ml of phosphate buffer (pH 6.8) was added and the steam distillate was collected into a 500 ml volumetric flask containing 250 ml dichloromethane, aqueous and organic layers were separated by using a separating funnel. The organic layer was treated with 50 ml of tartaric acid (1 M) to remove nicotine and was passed over anhydrous sodium sulphate. The final solution was concentrated to 1 ml for analysis (Wu *et al.*, 1992).

The GC-MS analysis was carried out using a QP 2010 Plus GC-MS system equipped with AOC - 20i auto sampler (Single quadrupole, Shimadzu Corporation, Kyoto, Japan). A ZB-5 MS (5% Phenyl, 95% Dimethyl polysiloxane) (Zebron™ – Phenomenex, USA) capillary column of 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness was used. The column oven temperature was programmed to rise from an initial temperature of 60 °C (hold for 1 min) to 140 °C (hold for 5 min) @ 6 °C/min, from 140 °C to 180 °C (hold for 5 min) @ 6 °C/min and to a final temperature of 210 °C @ 6 °C/min, the final temperature was held for 14 min with a total run time of 50 min. Helium was used as the carrier gas with a flow rate of 1 ml/min. The inlet and interface temperatures were kept at 250 °C. The Electron Ionisation (EI) source was operated at 200°C and the quadrupole temperature was 150 °C. All samples were analysed in scan mode with a mass range of 50 to 500 units. One micro liter (µl) of the sample was injected in split less mode by the auto sampler. The obtained peaks were identified using US National Institute of Standards and Technology (NIST) standard mass spectral library database. As authentic standards of the

compounds are not available for quantification, the area normalization method was adopted and the proportion of a particular compound in the total neutral volatile fraction was calculated.

RESULTS AND DISCUSSION

Nicotine, sugars and chlorides in the leaf are important chemical quality parameters in tobacco and it is inferred from the data of two seasons (Table 1) that the levels of nicotine (2.86 - 4.92%) was high, reducing sugars (0.51 - 0.85%) and chlorides (0.23 to 0.34%) were low. Umamaheswara Rao and Subba Rao (1992) described HDBRG tobacco as a nicotine booster used in cigarette blends because of higher nicotine content (4.49 – 5.48%) with lower levels of reducing sugars (1.70 – 1.86%) and chlorides (0.43 -0.55%). Deo Singh *et al.* (2003) reported that the sun-cured HDBRG tobacco was rich in tan to brown colour, medium to heavy body and broader leaf with nicotine (1.75 – 3.50%), reducing sugars (0.90 – 1.60%) and chlorides (0.75 – 1.50%). Genetics, environment and cultural practices have a profound influence on the nicotine levels in tobacco (Gopalachari and Gopinath, 1965). High nicotine and low reducing sugars could be attributed to the higher dose of nitrogen (150 kg/ha) applied and topping in the cultivation of HDBRG tobacco (Prasad Rao, 2005). In FCV tobacco, generally, the carbohydrate fraction was more (reducing sugars: 8.6 – 27.0%) when compared to the air-cured burley tobacco which contains lower levels of free sugar (Andersen and Litton, 1975). As HDBRG tobacco is used in cigarette blends, it is desirable to have lower levels of chlorides and the beneficial effects of small amounts in fertilizer are also reported (McCants and Woltz, 1967).

Solanesol content in the samples varied from 1.60 to 2.80% with mean values of 1.97 and 2.46% for the seasons 2009-10 and 2010-11, respectively and the data (Table 1) were consistent with the report of Phani Kiran *et al.* (2008) that HDBRG tobacco grown in the Guntur tract could be a rich source of solanesol (Range: 0.50 – 3.75%; Mean: 1.75%), both the maximum and mean values being the highest among different types of tobacco grown in the country. This fact could be attributed to genotype, growing conditions and agronomic practices which have profound influence on the solanesol content in the leaf at various growth stages of the plant, in particular, (1) cultivation under conserved moisture, (2) higher nitrogen fertilization and (3) topping could be the plausible reasons for higher solanesol in HDBRG. According to Court *et al.* (1984), nitrogen fertilization increased solanesol in flue-cured tobacco. Burton *et al.* (1989) investigated the factors influencing solanesol content in Burley tobacco and reported the following findings: (1) five-fold difference observed in solanesol concentration among genetic lines (0.53 – 3.10%), (2) growing season contributed to a ten-fold difference of solanesol for certain tobacco genotypes (0.60 – 6.00%), (3) soil-moisture deficits enhanced solanesol concentration (3.90%) at least four-fold and irrigation of the stressed tobacco decreased the solanesol level (1.00%), (4) solanesol concentration increased dramatically after topping for the top stalk position (3.80%) and there were marginal increases for the bottom and middle stalk positions and (5) nitrogen fertilization had only a minimal influence on solanesol concentration (2.20 - 3.00%). Solanesol content in various types of tobacco grown in different agro-ecological situations in India ranged from 0.09 to 3.18 % (Narasimha Rao and Prabhu, 2005).

In respect of the inorganic constituents (Table 2), the levels of total nitrogen (2.88 – 3.82%), potassium (0.67 – 1.67%) and phosphorus (0.17 – 0.23%) were similar to the levels reported in the literature for other air-cured/sun-cured tobaccos. Deo Singh (2003) reported higher total nitrogen content (1.50 – 2.50%) in HDBRG tobacco. A study on the effect of genotype and method of curing of chewing tobacco grown in Tamil Nadu (Siva Raju *et al.*, 2012) revealed the following trends in different sun-cured varieties: total nitrogen (Range: 2.73 – 3.02%; Mean: 2.90%), potassium (Range: 0.88 – 1.45%; Mean: 1.28%) and phosphorus (Range: 0.17 – 0.22%; Mean: 0.20%). Tso (1999) reported that in the case of samples from Burley culture, the nutrient composition was: total nitrogen (2.15 – 4.85%), potassium (1.05 – 5.40%) and phosphorus (0.14 – 0.45%). According to Leffingwell (1999), the nitrogen, potassium and phosphorus contents in Burley type were 3.96, 4.33 and 0.25%, respectively.

Swain *et al.* (1961) reported that the major portion of the PEE was resinous material, the others being paraffins, polyenes, esters, solanesol,

sterols, tocopherols and fatty acids. It is inferred from the data on lipid fractions (Table 3) that the total hexane solubles or PEE content in HDBRG tobacco is high (~ 10%) and solanesol accounted for ~ 25% of the extract. The non-polar and polar fractions accounted for 5.8 – 8.3% and 1.1 – 2.1% of the hexane extractives, respectively. Court *et al.* (1984) reported that nitrogen fertilization increased hexane extracts in flue-cured tobacco. In the case of sun-cured chewing tobacco varieties grown in Tamil Nadu, PEE levels varied from 5.90 to 7.80% with a mea value of 6.97% (Siva Raju *et al.*, 2012). Gangadhar *et al.* (2011) reported significant positive correlations of nicotine with solanesol and PEE and PEE with solanesol. Further investigations were initiated for characterization of the non-polar and polar fractions employing techniques like GC-MS and LC-MS.

In the neutral volatile fraction, neophytadiene, megastigmatrienone isomers, duvatrienediol, thunbergol, dibutylphthalate, solanone, (E,E)-farnesylacetone, 4-oxo-beta-isodamascol and phenylacetaldehyde accounted for ~96% of the compounds. Nerolidol, rishitin, neryl acetone, (12z)-abienol, phenylethyl alcohol, indole, viridiflorol and 3-hydroxysolavetivone, â-damascone and 14-labdien-8-ol are the other important flavour compounds identified (Table 4). It is inferred from the data that presence of higher levels of these compounds could be attributed to higher nitrogen and nicotine contents in HDBRG leaf thus indicating their positive impact on the smoke flavour. Shi *et al.* (2009) reported that flavour quality of tobacco is highly correlated with nicotine and total nitrogen contents. Higher neophytadiene proportion (~ 53%) in this tobacco is an important factor as the compound has been suggested to be a tobacco flavour enhancer and is considered as a flavour carrier by entrapping volatiles in the tobacco smoke aerosol (Leffingwell and Leffingwell, 1988). According to Fujimori *et al.* (1978), the medium-range boiling point fraction of burley tobacco was composed of neophytadiene-related compounds: 46.2%, degradation products of carotenoids (megastigmatrienones, â-damascene etc.): 7.7%, degradation products of thunberganoids (solanone, duvatrienediol etc.): 3.6% and mono and sesquiterpenoids (solavetivone etc.): 4.7%.

It is concluded that HDBRG tobacco has higher levels of nicotine & total N, lower levels of reducing sugars, chlorides & phosphorus and medium level of potassium. Also, higher levels of hexane soluble fraction and solanesol in the fraction were recorded. Higher levels of neutral volatile compounds in HDBRG tobacco could be attributed to higher levels of nitrogen and nicotine in leaf thus indicating their positive impact on the smoke flavour.

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Table 1: Solanesol and chemical quality characters of HDBRG tobacco

Sample	Solanesol (%)		Nicotine (%)		Red. sugars (%)		Chlorides (%)	
	2009-10	2010-11	2009-10	2010-11	2009-10	2010-11	2009-10	2010-11
1	1.60	2.05	3.63	3.97	0.85	0.78	0.28	0.31
2	1.60	2.10	3.68	3.98	0.75	0.78	0.25	0.34
3	1.60	2.05	3.59	3.96	0.78	0.71	0.26	0.29
4	1.65	2.50	2.86	4.86	0.52	0.69	0.23	0.25
5	1.65	2.50	2.93	4.87	0.53	0.72	0.25	0.25
6	1.65	2.50	2.88	4.92	0.51	0.70	0.28	0.28
7	2.70	2.80	3.07	3.73	0.61	0.73	0.35	0.25
8	2.65	2.80	3.02	3.76	0.61	0.78	0.34	0.27
9	2.60	2.80	3.07	3.73	0.60	0.81	0.34	0.27
Mean	**1.97**	**2.46**	**3.19**	**4.20**	**0.64**	**0.74**	**0.29**	**0.28**
SD	**0.51**	**0.32**	**0.34**	**0.52**	**0.12**	**0.04**	**0.05**	**0.03**
CV (%)	**26.11**	**13.02**	**10.65**	**12.48**	**19.34**	**5.82**	**15.79**	**10.97**
SEm±	**0.17**	**0.11**	**0.113**	**0.175**	**0.041**	**0.014**	**0.015**	**0.010**
CD (P=0.05)	**0.004**	**NS**	**0.03**	**NS**				

Table 2: Inorganic constituents in HDBRG tobacco

Sample	Nitrogen (%)		Potassium (%)		Phosphorus (%)	
	2009-10	2010-11	2009-10	2010-11	2009-10	2010-11
1	3.12	3.36	1.47	1.20	0.18	0.20
2	3.11	3.28	1.32	1.20	0.18	0.22
3	3.08	3.27	1.25	1.22	0.17	0.22
4	2.91	3.44	1.67	0.87	0.21	0.21
5	2.88	3.56	1.70	0.87	0.22	0.20
6	3.12	3.56	1.70	0.82	0.22	0.19
7	3.78	3.60	0.80	0.70	0.22	0.22
8	3.82	3.60	0.80	0.67	0.23	0.21
9	3.68	3.68	0.78	0.70	0.23	0.21
Mean	**3.28**	**3.48**	**1.28**	**0.92**	**0.21**	**0.21**
SD	**0.37**	**0.15**	**0.40**	**0.23**	**0.02**	**0.01**
CV (%)	**11.41**	**4.33**	**31.01**	**25.03**	**11.35**	**5.05**
SEm±	**0.12**	**0.05**	**0.13**	**0.08**	**0.008**	**0.004**
CD (P=0.05)	**NS**	**NS**	**NS**			

Table 3: Lipid fractions of HDBRG tobacco

Fraction	Range	Mean (2009-10)	Mean (2010-11)
Total hexane solubles (%)	7.57 – 11.94	9.72	10.50
Non-polar fraction (%)	5.75 – 8.31	6.87	6.95
Polar fraction (%)	1.10 – 2.10	1.27	1.97
Solanesol content in the hexane extract (%)	20.80 – 25.48	24.81	23.66

Table 4: Composition of the neutral volatile fraction of HDBRG tobacco

Compound	Per cent of neutral volatile fraction	Compound	Per cent of neutral volatile fraction
Neophytadiene	52.8	Rishitin	0.7
Megastigmatrienones	21.0	Neryl Acetone	0.5
Duvatriendiol	12.0	(12z)-Abienol	0.3
1-Nonadecene	8.0	Phenylethyl Alcohol	0.3
Thunbergol	3.8	Viridiflorol	0.2
Dibutyl Phthalate	2.0	Indole	0.2
(E,E)-Farnesylacetone	1.6	3-Hydroxysolavetivone	0.1
Solanone	1.3	14-Labdien-8-Ol	0.05
4-Oxo-Beta-Isodamascol	0.9	Beta Damascone	0.03
Nerolidol	0.7		

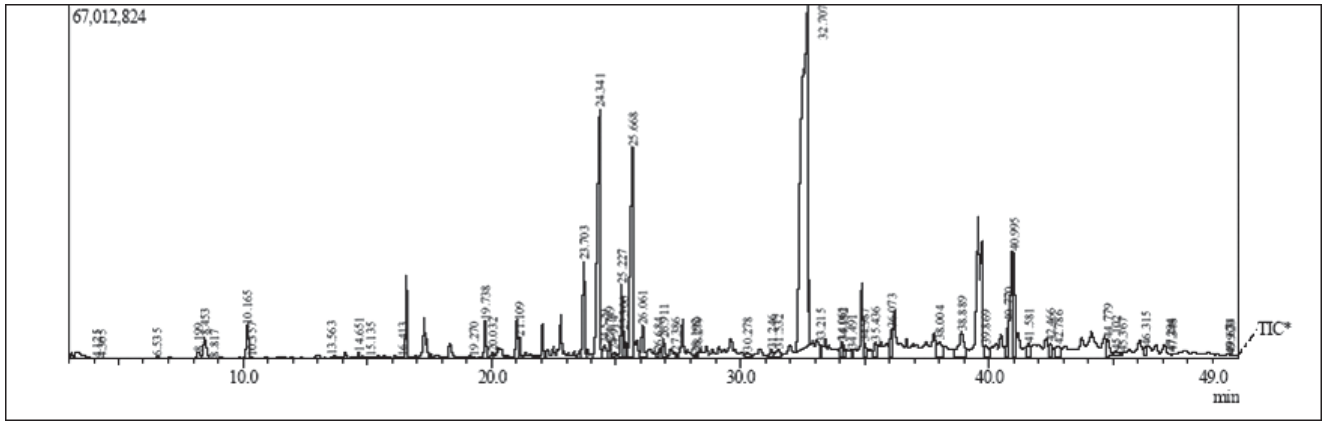

Fig. 1: A typical GCMS chromatogram of neutral volatiles compounds

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