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Characterization and Identification of Dolichos Bean (*Lablab purpureus* L. sweet) Recombinant Inbred Lines (RIL) with High Pod Yield and High Pod Fragrance

Uday Kumar H. R.[1*], Byre Gowda M. [2], Ramesh S.[1] and Vasundhara M.[3]

[1]Department of Genetics and Plant Breeding, University of Agricultural Sciences (UAS), Bengaluru, Karnataka, India

[2]All India Coordinated Research Project on Pigeonpea, UAS, Bengaluru, Karnataka, India

[3]Department of Horticulture, UAS, Bengaluru, Karnataka, India

*Corresponding Author E-mail: udayhr7096@gmail.com

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ABSTRACT

Pod fragrance in dolichos bean is being routinely assessed by organoleptic (smelling) means. Phenotyping pod fragrance by organoleptic means is highly subjective. The relativity and subjectivity associated with organoleptic means of phenotyping pod fragrance could be overcome by objective means, that is by analyzing and quantifying trans 2-Dodecenoic acid and trans 2-Tetradecenoic acids, the key fatty acids (FA) in the pod exudates of dolichos bean. An attempt was made to characterize recombinant inbred lines (RIL) population derived from two connected crosses for pod fragrance by quantifying the two key FA in pod exudates and to explore correspondence between organoleptic and objective means of phenotyping pod fragrance. The variability among RIL was substantial for both the FA as indicated by the estimates of absolute and standardized range and PCV. The positively skewed platykurtic distribution of RIL population for both the key FA indicated that the pod fragrance is controlled by fewer genes with complementary epistasis. The concentration of the two FA in pod exudates of the best 10 RILs of both the populations were higher than that of the mean by at least 60 per cent. The RIL 3-3 was one of the best 10 RIL with respect to concentration of trans 2-Tetradecenoic acid and promising for fresh pods plant^{-1}, fresh pod yield plant^{-1}, and fresh seed yield plant^{-1}. The significance of mean concentration of the two key FA of pod exudates of RIL classified under high, medium and low pod fragrance groups based on organoleptic means by, 'F' test indicated good correspondence between objective and organoleptic means and reliability of screening RIL population by organoleptic means.

Key words: *Fatty acids, Pod fragrance, Trans-2-dodecenoic acid, Trans-2-tetradecenoic acid*

INTRODUCTION

Dolichos bean var. lignosus is one of the important legumes grown in India. Karnataka contributes >90 *per cent* of total dolichos bean production in India. It is a multipurpose legume crop used as vegetable, pulse[1,11] forage[7].

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While fresh beans are used as a vegetable, dried whole seed and split dhal are used in various food preparations. For use as a vegetable, fresh pods with immature beans are the harvestable economic product in dolichos bean. Fresh pod walls secret oily substances that emit characteristic fragrance. Pod fragrance is one of the 'farmers' and 'consumers' preferred traits in dolichos bean varieties[1,11,13]. The fresh pods with high pod fragrance fetch premium price in the market. Being predominantly self-pollinated crop, development of high fresh pod yielding pure-line varieties with high pod fragrance is one of the objectives of breeding dolichos bean.

Pod fragrance in germplasm accessions/ segregating populations/ advanced breeding lines is being routinely assessed by organoleptic (smelling) means using a panel of analysts. As analysts differ in their ability to detect pod fragrance and their sensitivity will be reduced if there are subtle differences among the individuals of breeding populations, phenotyping pod fragrance by organoleptic means is highly subjective. Pod fragrance in dolichos bean has been attributed to Trans 2-Dodecenoic acid and trans 2-Tetradecenoic acids in the pod exudates[5,13]. Based on similar kinds of chemical composition analysis, Dunemann *et al.*[2] in apple and Eduardo *et al*.[3], in peach attributed fruits aroma to various volatile organic compounds (VOC) and mapped quantitative traits loci (QTLs) controlling variation for respective VOCs. Taking cues from these studies, the relativity and subjectivity associated with organoleptic means of phenotyping pod fragrance could be overcome by objective means, that is by analyzing and quantifying trans 2-Dodecenoic acid and trans 2-Tetradecenoic acids, the key fatty acids (FA) in the pod exudates of dolichos bean. The objectives of the present investigation were to (1) characterize recombinant inbred lines (RIL) population derived from two connected crosses for pod fragrance by quantifying the two key FA in pod exudates and (2) to explore correspondence between organoleptic and objective means of phenotyping pod fragrance.

MATERIAL AND METHODS

Material:

The material consisted of 95 F_9 RILs derived from HA 4 × CPI 31113, 58 F_9 RILs derived from HA 4 × CPI 60125 and three check entries [HA 3, HA 4 and kadalavare (KA)] being maintained at All India Co-ordinated Research Project (AICRP) on pigeon pea, University of Agricultural Sciences (UAS), Bengaluru, India. The seed coats of these RILs differed in thickness. HA 3 and HA 4 are high yielding photoperiod insensitive pure-line varieties with determinate growth habit and medium and high pod fragrance respectively. CPI 31113, CPI 60125 and KA are photoperiod sensitive landrace varieties with indeterminate growth habit and medium pod fragrance.

Methods:

Field evaluation of RILs: The seedlings of all the RILs and the checks were raised in polythene covers and maintained for 15-20 days for proper rooting. Subsequently, the seedlings of RIL populations and those of the three check entries were transplanted in experimental plot following augmented design[5] in seven compact blocks during 2016 rainy season at Zonal Agricultural Research Station (ZARS), UAS, Bengaluru. Each block consisted of 20-25 RILs, three checks and two border entries. The seedlings of each entry were transplanted in a single row of 2.5 m length by maintaining 0.2 m spacing between seedlings, with a row spacing of 0.45 m. A basal dose of 25:50:25 Kg ha^{-1} of NPK (nitrogen:phosphorous:potassium) was applied to the experimental plots. Recommended management practices were followed during the crop-growing period to raise a healthy crop.

Phenotyping RILs for pod fragrance: Pod fragrance was assessed using (1) organoleptic and (2) objective methods. In organoleptic method, five fresh pods harvested from each RIL were offered to the panel to assess the fragrance by smelling through relative rating. The pod fragrance of RILs was assessed by averaging relative rates given by a panel of three persons involving a student, farm labor

and research fellow. Based on the opinion of the panel, RIL were classified as high, medium and low fragrant groups. In objective method, pod fragrance was quantified in three steps, (1) preparation of the pod exudates' samples, (2) esterification of pod exudates and (3) fatty acid profiling of esterified pod exudates using gas chromatographic mass spectrometry (GC-MS) (Fig. 1).

Pod exudates' sample preparation: The exudates of the pods borne on all the RILs and the three checks were collected by wiping the surface of pods with the filter paper pieces (about 3 × 2 cm). Then, pieces of filter paper with the absorbed pod exudates were quickly transferred into a bottle containing 25 ml petroleum ether. The bottle containing the petroleum ether was shaken well and then decanted from the bottle. The filter paper pieces were washed using 50 ml methanol. Both the petroleum ether solution and methanol washings were combined and filtered. After that, the solvents were removed from a mixture under aspirator pressure at 40-50°c, leaving pleasant smelling oil extract. The oil extract was subjected to esterification.

Esterification of pod exudates using GC-MS: About 500 mg of oil extract was transferred to 250 ml flat-bottom flask and then 92 ml methanol and 8 ml Conc. H_2SO_4 was added to oil extract. After that, mixture of 500 mg oil extract + 92 ml methanol + 8 ml Conc. H_2SO_4 was kept on heating mantle and connected to water condenser reflux for 90 min. Then oil extract + methanol + Conc. H_2SO_4 mixture was cooled to room temperature and transferred to separator funnel. To wash-out the acid content from the sample, 100 ml diethyl ether + 100 ml distilled water was added into the funnel and then diethyl ether layer was collected. This process was continued for 4-5 times. Subsequently, diethyl ether layer was passed through anhydrous sodium sulphate (Na_2SO_4) and collected in a 500 ml beaker. Finally, solvent was evaporated on water bath and concentrated to 1 ml. From the 1 ml concentrated solvent, 1-2 µl was used to inject in to GC column.

Fatty acid profiling of pod exudates using GC-MS analysis: An Rtx-5 column with a length of 30m, internal diameter 0.25mm, and particle size of 0.25 micron meter was installed in the GC. One µL of esterified oils from the pod exudates of all the RILs were fractionated in GC-MS to estimate the composition of FA.

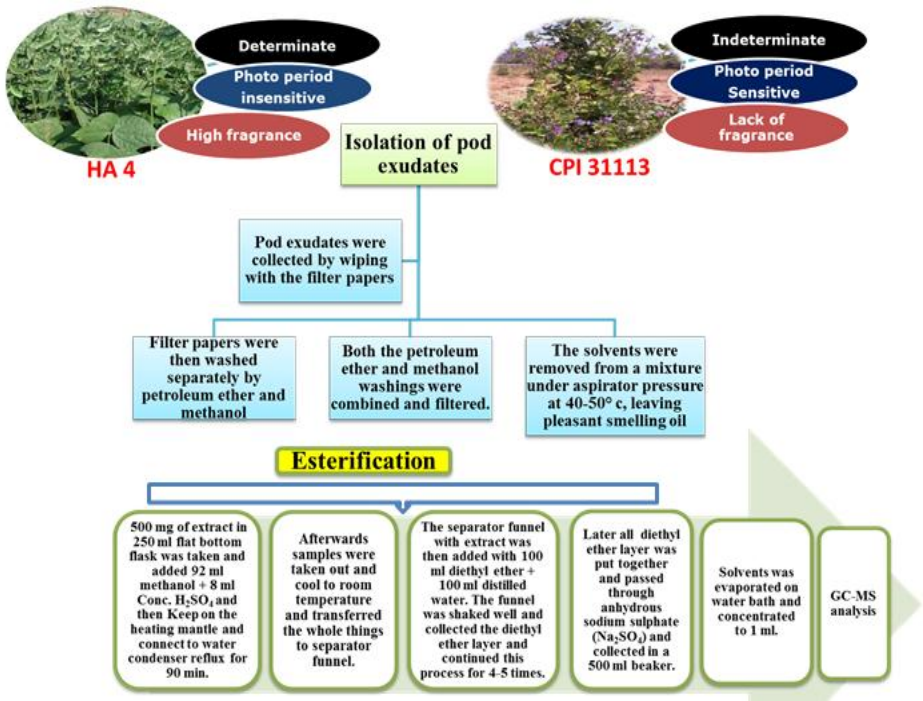

Fig. 1: Schematic representation of sample preparation for GC-MS analysis for pod fragrance in dolichos bean

Statistical analysis

The data obtained from GC-MS analysis on two key FA (trans-2-Dodecenoic acid and trans-2-Tetradecenoic acid) responsible for pod fragrance were used to estimate the descriptive statistics such as, mean, absolute and standardized range, phenotypic coefficient of variation (PCV), skewness and kurtosis. The mean ($\bar{X}$) was estimated as, $(\bar{X}) = \sum_{i=0}^{n} \frac{X_i}{N}$, where, $\bar{X}$=Population mean, X_i = value of i^{th} individual, N = Number of individuals; The absolute range was estimated as the difference between minimum and maximum concentration of the two key FA of pod exudates of RIL; standardized range (SR) was estimated as, SR = (Max – Min)/ $\bar{X}$; phenotypic coefficient variation was estimated as,

$$\text{PCV}(\%) = \frac{\sqrt{Phenotypic\ variance}}{General\ mean} \times 100$$

The PCV was classified into low (0 – 10 %), moderate (10.1 % - 20 %) and high (> 20 %)[9]. The skewness and kurtosis were estimated using statistical analysis option available in Microsoft excel.

Interpretation of skewness and kurtosis: The skewed distribution of a trait in general suggests that the trait is under the control of non-additive gene action, especially epitasis and is influenced by environmental variables[6,8,10]. Positive skewness is caused by complementary gene interactions, while negative skewness is caused by duplicate gene interactions predominantly in the same directions[12]. Complete ambi-directional epistasis however produces kurtosis while distributions stays symmetrical around mean[8]. The genes controlling the trait with skewed distribution tend to be predominantly dominant irrespective of whether they have increasing or decreasing effect on the expression of trait. Kurtosis, the fourth-degree statistic depicts degree of peakedness of distribution of RIL. 'Negative' kurtosis indicates 'platykurtic' distribution; 'positive' kurtosis indicates 'leptokurtic' distribution; near 'zero' kurtosis indicates 'mesokurtic' distribution.

Correspondence between objective and subjective means of assessing pod fragrance: The significance/otherwise of differences in the mean contents of the two key FA in pod exudates of RIL classified as high, medium and low groups based on pod fragrance by organoleptic means were examined using 'F-test' implemented using statistical analysis option available in Microsoft excel. Significance/otherwise of 'F' test indicate good/poor degree of correspondence between objective means (by quantifying the two key FA) and subjective means (by organoleptic method) of assessing pod fragrance.

RESULTS AND DISCUSSION

The pod fragrance has been attributed to occurrence of two dominant fatty acids (FA) such as Trans 2-Dodecenoic acid and Trans 2-Tetradecenoic acids[5,13]. Thus, these two FA were quantified in 95 and 58 HACPI 3 and HACPI 6-derived RIL populations respectively. Of the two key FA, while trans-2-Dodecenoic acid varied from 0.12 to 35.84 *per cent* with a mean of 11.09, trans-2-Tetradecenoic acid varied from 0.12 to 32.21 *per cent* with a mean of 14.08 in the HACPI 3-derived RIL population, whereas in HACPI 6-derived RIL population, trans-2-Dodecenoic acid varied from 0.11 to 19.68 *per cent* with a mean of 5.92, trans-2-Tetradecenoic acid varied from 0.28 to 31.92 *per cent* with a mean of 9.45 (Table 1). The variability among RIL was substantial for both the FA as indicated by the estimates of absolute and standardized range and PCV. The positively skewed platykurtic distribution of RIL populations for both the key FA (Table 1 and, Fig. 2a&2b and 3a&3b) indicated that the pod fragrance is controlled by fewer genes with complementary epistasis. Intense selection is expected to result rapid improvement in pod fragrance. Sizable proportion of RIL transgressed their parents for both FA.

The concentration of the two FA in pod exudates of the best 10 RILs of both the

populations were higher than that of the mean by at least 60 *per cent* (Table 2a & 2b and 3a & 3b). The RIL 3-3 was one of the best 10 RIL with respect to concentration of trans 2-Tetradecenoic acid. This RIL was also promising for fresh pods plant^{-1}, fresh pod yield plant^{-1}, and fresh seed yield plant^{-1} (Table 3a). The superiority of this RIL needs to be confirmed through multi-location trials for arriving at a decision for use as a variety (if found consistently superior to the checks across locations and year) or use as a traits-donar parents for developing improved varieties. The significance of mean concentration of the two key FA of pod exudates of RIL classified under high, medium and low pod fragrance groups based on organoleptic means as indicated by, 'F' test (Table 4) indicated good correspondence between objective and organoleptic means and reliability of screening RIL population by organoleptic means. Being a rapid and cost-effective, organoleptic means could be routinely used for screening large germplasm and breeding populations for shortlisting the most promising ones for pod fragrance. For confirmation of the shortlisted high fragrant germplasm accessions and breeding population, objective means, that is by quantifying the concentration of the two key FA need to be used. Also for purpose of mapping and subsequent cloning of QTL controlling pod fragrance, the objective means of screening is suggested as the most appropriate approach.

Table 1: Descriptive statistics of the two key fatty acids responsible for pod fragrance in dolichos bean

The key fatty acids responsible for pod fragrance		Mean ± Se	Range		Standardized Range	PCV %	Skewness	Kurtosis
			Max	Min				
Trans 2-Dodecenoic acid (%)	HACPI 3	11.09 ± 0.83	35.84	0.12	3.22	73.35	0.90	0.43
	HACPI 6	5.92 ± 0.60	19.68	0.11	3.30	77.53	0.90	0.71
Trans 2-Tetradecenoic acid (%)	HACPI 3	14.08 ± 0.84	32.21	0.12	2.27	58.58	0.17	-0.87
	HACPI 6	9.45 ± 0.96	31.92	0.28	3.34	77.98	1.04	0.65

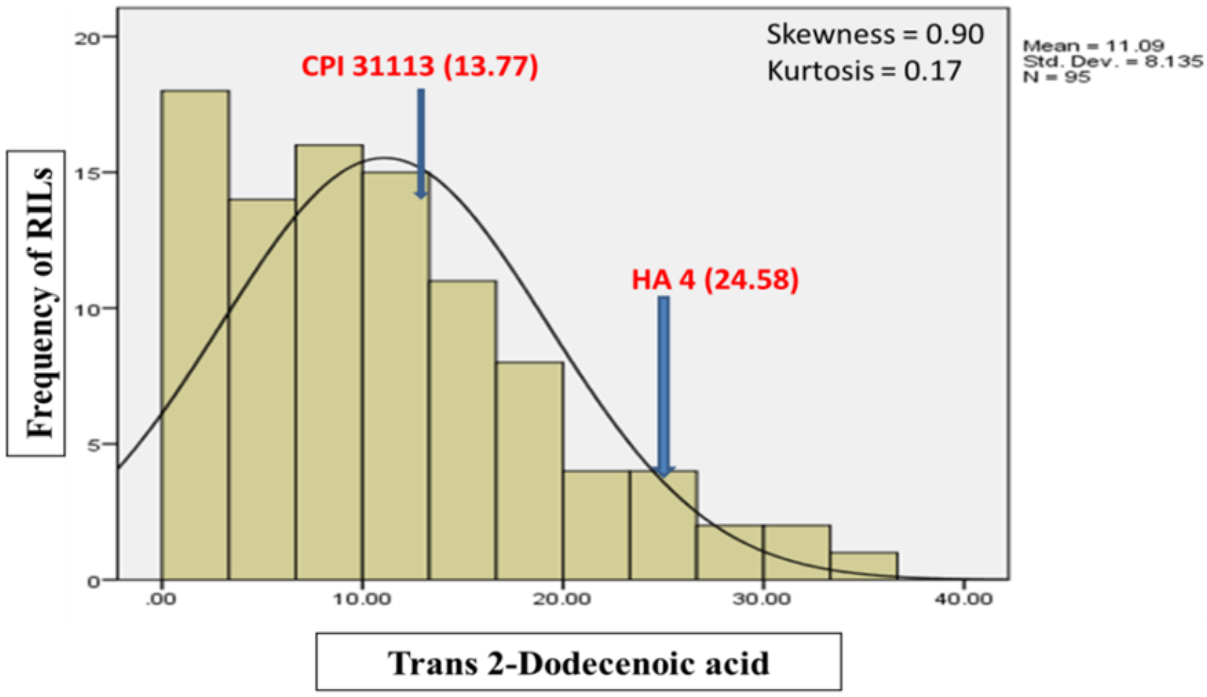

Fig. 2a: Distribution of HACPI 3-derived RIL for Trans 2-Dodecenoic acid of pod exudates

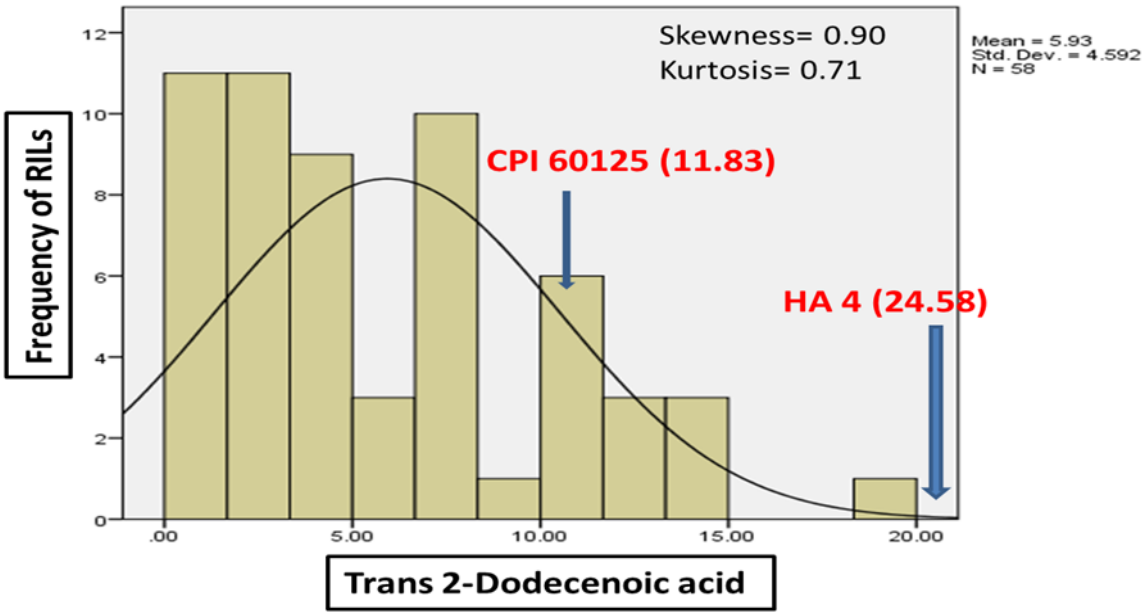

Fig. 2b: Distribution of HACPI 6-derived RIL for Trans 2-Dodecenoic acid of pod exudates

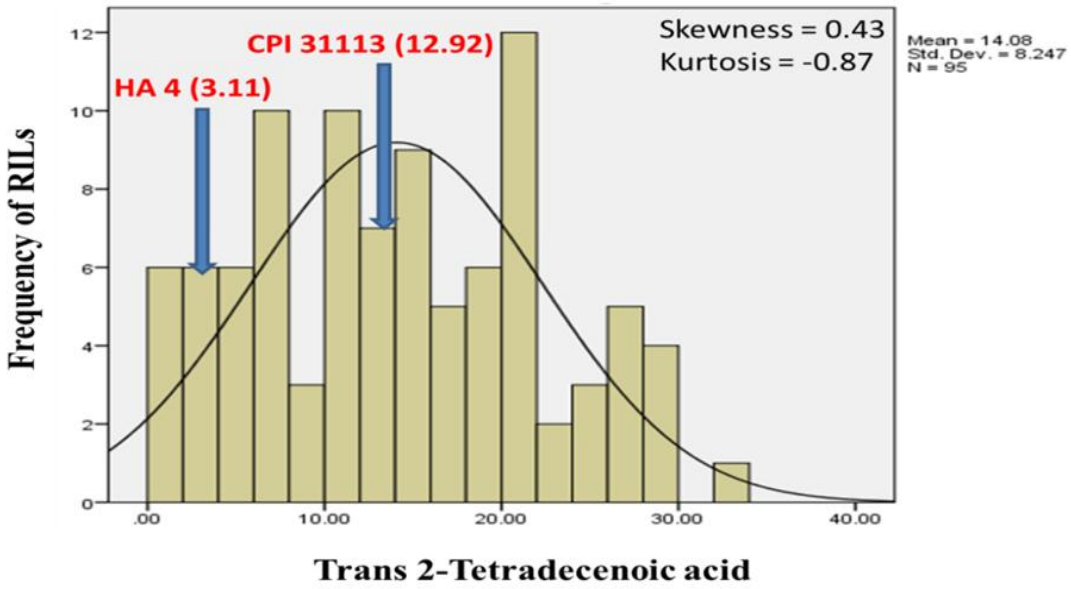

Fig. 3a: Distribution of HACPI 3-derived RIL for Trans 2-Tetradecenoic acid of pod exudates

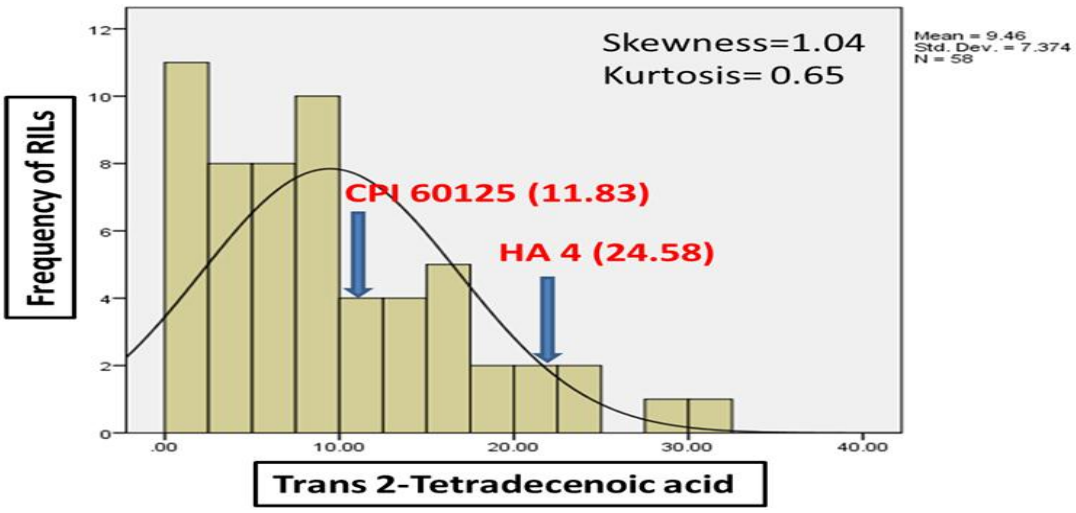

Fig. 3b: Distribution of HACPI 6-derived RIL for Trans 2-Tetradecenoic acid of pod exudates

Table 2a: The best 10-RIL derived from HACPI 3 with higher concentration of Trans 2-Dodecenoic acid

RIL identity	Trans 2-Dodecenoic acid (%)	Trans 2-Tetradecenoic acid (%)	Days to 50% flowering	Fresh pods plant^{-1}	Fresh pod yield plant^{-1}	Fresh seed yield plant^{-1}	Dry seed yield plant^{-1}
RIL 3-22	26.01	24.28	65.00	57.37	100.53	48.77	16.41
RIL 3-26	32.07	16.79	42.50	60.31	221.28	101.16	39.97
RIL 3-50	35.84	27.38	57.00	11.99	21.00	17.73	06.84
RIL 3-53	26.41	08.79	65.50	19.78	23.21	13.97	10.99
RIL 3-65	22.00	14.08	79.50	8.02	21.23	09.44	01.38
RIL 3-68	26.36	16.78	52.50	21.58	05.28	08.70	08.62
RIL 3-83	22.68	15.15	72.50	54.78	59.25	46.95	18.69
RIL 3-98	19.63	15.23	71.00	23.63	9.43	06.65	3.75
RIL 3-124	26.69	09.68	57.50	31.11	99.58	51.57	32.66
RIL 3-130	27.50	5.63	58.00	18.23	19.27	08.83	12.45
Parents							
HA 4	24.58	3.11	45.13	36.47	59.33	29.66	21.38
CPI 31113	13.77	12.92	89.00	43.50	46.80	22.40	14.80
Checks							
HA 3	05.24	15.62	49.91	34.78	59.71	28.24	17.67
Kadalavare	15.31	06.67	64.58	43.34	70.94	35.67	27.25

Table 2b: The best 10-RIL derived from HACPI 3 with higher concentration of Trans 2-Dodecenoic acid

RIL identity	Trans 2-Dodecenoic acid (%)	Trans 2-Tetradecenoic acid (%)	Days to 50% flowering	Fresh pods plant^{-1}	Fresh pod yield plant^{-1}	Fresh seed yield plant^{-1}	Dry seed yield plant^{-1}
RIL 6-202	11.33	5.87	51.62	29.51	42.24	10.76	8.26
RIL 6-205	19.68	14.44	42.97	22.95	31.65	13.33	8.34
RIL 6-220	13.00	31.92	36.42	34.62	47.04	27.98	14.94
RIL 6-261	13.18	22.34	68.93	17.85	26.69	8.61	5.07
RIL 6-274	14.07	14.25	60.51	30.97	32.62	11.75	6.24
RIL 6-276	11.25	6.08	86.46	31.00	48.96	18.08	10.56
RIL 6-316	11.41	14.52	60.51	38.99	55.61	24.20	10.75
RIL 6-321	12.45	16.52	55.98	28.78	28.90	28.17	13.49
RIL 6-354	13.88	7.22	37.97	16.39	27.66	16.60	7.56
RIL 6-356	13.71	8.88	69.65	20.03	51.98	28.49	14.19
Parents							
HA 4	24.58	03.11	45.13	36.47	59.33	29.66	21.38
CPI 60125	4.88	11.83	91.00	46.50	56.30	26.50	18.10
Checks							
HA 3	05.24	15.62	49.91	34.78	59.71	28.24	17.67
Kadalavare	15.31	06.67	64.58	43.34	70.94	35.67	27.25

Table 3a: The best 10-RIL derived from HACPI 3 with higher concentration of Trans 2-Tetradecenoic acid

RIL identity	Trans 2-Tetradecenoic acid (%)	Trans 2-Dodecenoic acid (%)	Days to 50% flowering	Fresh pod plant^{-1}	Fresh pod yield plant^{-1}	Fresh seed yield plant^{-1}	Dry seed yield plant^{-1}
RIL 3-3	27.57	12.43	55.50	69.83	200.40	99.68	31.24
RIL 3-13	22.59	14.88	74.50	50.45	63.54	33.00	12.69
RIL 3-22	24.28	26.01	65.00	57.37	100.53	48.77	16.41
RIL 3-31	29.69	14.14	61.00	26.88	74.86	24.84	16.17
RIL 3-50	27.38	35.84	57.00	11.99	21.00	17.73	06.84
RIL 3-52	22.31	16.09	56.50	08.60	17.11	14.02	05.48
RIL 3-67	32.21	10.93	70.50	15.84	33.39	22.22	08.66
RIL 3-71	26.34	15.51	45.00	36.94	175.23	12.18	04.92
RIL 3-79	25.05	18.70	75.50	22.38	30.76	11.86	05.60
RIL 3-91	28.71	08.86	49.00	48.72	49.21	28.41	13.82
Parents							
HA 4	24.58	03.11	45.13	36.47	59.33	29.66	21.38
CPI 31113	13.77	12.92	89.00	43.50	46.80	22.40	14.80
Checks							
HA 3	05.24	15.62	49.91	34.78	59.71	28.24	17.67
Kadalavare	15.31	06.67	64.58	43.34	70.94	35.67	27.25

Table 3b: The best 10-RIL derived from HACPI 6 with higher concentration of Trans 2-Tetradecenoic acid

RIL identity	Trans 2-Tetradecenoic acid (%)	Trans 2-Dodecenoic acid (%)	Days to 50% flowering	Fresh pod plant^{-1}	Fresh pod yield plant^{-1}	Fresh seed yield plant^{-1}	Dry seed yield plant^{-1}
RIL 6-189	22.71	10.21	51.96	23.68	33.34	15.64	8.67
RIL 6-220	31.92	13	36.42	34.62	47.04	27.98	14.94
RIL 6-238	20.66	7.53	41.75	12.01	24.25	12.36	5.38
RIL 6-250	16.95	7.93	65.06	26.60	50.18	25.85	10.49
RIL 6-261	22.34	13.18	68.93	17.85	26.69	8.61	5.07
RIL 6-264	27.93	8.3	68.55	26.60	56.73	31.81	24.91
RIL 6-308	24.3	3.92	44.17	26.60	18.58	4.71	3.62
RIL 6-321	16.52	12.45	55.98	28.78	28.90	28.17	13.49
RIL 6-332	18.62	5.41	53.96	45.55	32.82	16.32	7.16
RIL 6-352	19.78	7.57	51.79	35.35	33.33	25.66	11.05
Parents							
HA 4	24.58	03.11	45.13	36.47	59.33	29.66	21.38
CPI 60125	4.88	11.83	91.00	46.50	56.30	26.50	18.10
Checks							
HA 3	05.24	15.62	49.91	34.78	59.71	28.24	17.67
Kadalavare	15.31	06.67	64.58	43.34	70.94	35.67	27.25

Table 4: The mean concentration of the key fatty acids in pod exudates of RIL classified into high, medium and low pod fragrance based on organoleptic means

		Classification of RIL for pod fragrance based on organoleptic means						'F' statistic
		High		Medium		Low		
Descriptive statistics of the key FA responsible for pod fragrance		Mean	Range	Mean	Range	Mean	Range	
Trans 2-Dodecenoic acid (%)	HACPI 3	20.91	5.84-10.93	11.43	0.12-32.82	5.35	0.21-10.46	16.79**
	HACPI 6	11.27	3.92-19.68	6.15	1.25-13.71	4.19	0.25-14.07	8.19**
Trans 2-etradecenoic acid (%)	HACPI 3	20.19	9.68-32.21	14.81	0.31-29.69	8.90	0.12-20.44	8.23**
	HACPI 6	20.19	5.87-31.92	8.45	1.45-27.93	7.48	0.28-20.66	11.78**

**= Significant at P=0.01

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