Histochemical and biochemical analysis of major aroma compound (2-acetyl-1-pyrroline) in basmati and other scented rice (*Oryza sativa* L.)

A. B. Nadaf[1], S. Krishnan[2,*] and K. V. Wakte[1]

[1]Department of Botany, University of Pune, Pune 411 007, India
[2]Department of Botany, Goa University, Goa 403 206, India

Histochemical studies were carried out to localize the major aroma compound 2-acetyl-1-pyrroline (2AP) in mature rice caryopsis of basmati and other scented rice varieties. Among the histochemical reactions tried, the reagent 2,4-dinitrophenyl hydrazine was found to localize 2AP. The structure of 2AP includes a non-reactive pyrroline ring and a reactive methyl ketone group, which reacts with 2,4-dinitrophenyl hydrazine to produce an orange-red coloured compound, 2-acetyl-phenyl hydrazone. The reaction was confirmed in *Pandanus amaryllifolius* leaves, which is known to contain ten times higher concentration of 2AP than basmati rice, and also with the extracts of *P. amaryllifolius* in paper chromatogram. The biochemical analysis revealed that among the cultivars studied, the marketed basmati rice showed maximum 2AP content (0.061 ppm). Pusa basmati grown during the rainy season had 0.030 ppm 2AP followed by the local scented cultivar Ghansal (0.028 ppm). The non-scented variety Krishnahansa and Pusa basmati rice grown during summer do not show any presence of 2AP. This study may help in better understanding the biology of rice grain and improvement of rice quality.

Keywords: 2-Acetyl-1-pyrroline, basmati rice, histochemical and biochemical analysis, *Pandanus amaryllifolius*.

RICE is a major source of nutrition and more than half the world's population is dependent on rice as a staple food[1]. Scented rices are an important commodity worldwide and command premium prices over non-scented varieties. The Indian subcontinent has the 'natural gift' of basmati rice that has been accepted as the best scented, long and slender grain rice in the world markets and fetches high prices. In addition to basmati, many indigenous scented rice varieties are also grown that excel in aroma and cooking qualities. Most of these varieties are medium or short-grain varieties and hence are not traded at international level. However, in domestic markets, their demand is far greater than the long-grain ones.

More than 100 volatile compounds have been identified in basmati rice[1–4]. Among these compounds, Buttery *et al.*[5] have identified 2-acetyl-1-pyrroline (2AP) as the principal aroma compound. Later many studies have confirmed the presence of this compound in all the scented rice varieties. Many attempts were made in the past to develop techniques for its localization[6,7]. In the present study, histochemical localization of 2AP is reported along with biochemical analysis for some local scented rice varieties and Pusa basmati grown in two seasons, i.e. summer and rainy season.

For histochemical studies, seeds of the variety 'Tarori' basmati and non-scented variety 'Jaya' were obtained from ICAR Research Complex for Goa, Goa, India. The variety Jaya was taken as control. The seeds of both varieties were soaked overnight to soften the seed-coat. Next day, the seeds were manually de-husked and hand-cut transverse sections were obtained using a razor blade. Thin sections were passed through different alcohol grades (from 10, 20, 30, 50, 70, 90% and absolute alcohol). Then the sections were transferred to 2,4-dinitrophenyl hydrazine reagent in a cavity block and incubated in hot air oven at 60°C for 30 min. The reagent was prepared by dissolving 2 g of 2,4-dinitrophenyl hydrazine in 15 ml conc. sulphuric acid followed by adding 15 ml of 95% ethanol and diluting the solution to 150 ml with distilled water. Later, the sections were mounted in Canada balsam, and observed and photographed using Nikon E800 microscope with automatic exposure system under bright field mode. In order to cross-check the above reaction, sections of the leaves of *Pandanus amaryllifolius* Roxb. was used. For TLC analysis, 100 g of *P. amaryllifolius* leaves was distilled in 1 l distilled water and the volatile extract was extracted in diethyl ether. The extract in ether was condensed to 5 ml at 33 ± 2°C using temperature-controlled water bath. The condensed extract was loaded on TLC plate and sprayed with 2,4-dinitrophenyl hydrazine reagent. The colour development was observed.

For biochemical studies, the scented cultivated variety 'Pusa' basmati and non-scented variety 'Krishnahansa' were used. Both varieties were grown in two seasons (rainy and summer season) at ICAR Research Complex for Goa, Goa, India. For comparative analysis two local, scented, short-grain varieties were collected from farmers at Ajra, Maharashtra, India. In addition, marketed basmati rice (Kohinoor brand) was also used for analysis. Extraction of aroma compounds was done by steam distillation under reduced pressure using 200 g of whole, uncooked brown rice. Vapour containing extracted volatiles from rice was then condensed by passing it through a cooled condenser. The condensed extract was transferred into a 500 ml separating funnel and extracted twice, each time using 250 ml of dichloromethane. The organic layer was concentrated to 50 ml using a rotary evaporator under reduced pressure at a temperature of 26°C followed by drying with anhydrous sodium sulphate. The same extract was concentrated to a volume of 1 ml. The concentrated extract was left uncovered at room temperature and allowed to evaporate until its volume decreased to 0.2 ml. Next 1 µl of condensed sample was injected in GC for the analysis.

*For correspondence. (e-mail: skrish8@yahoo.com)

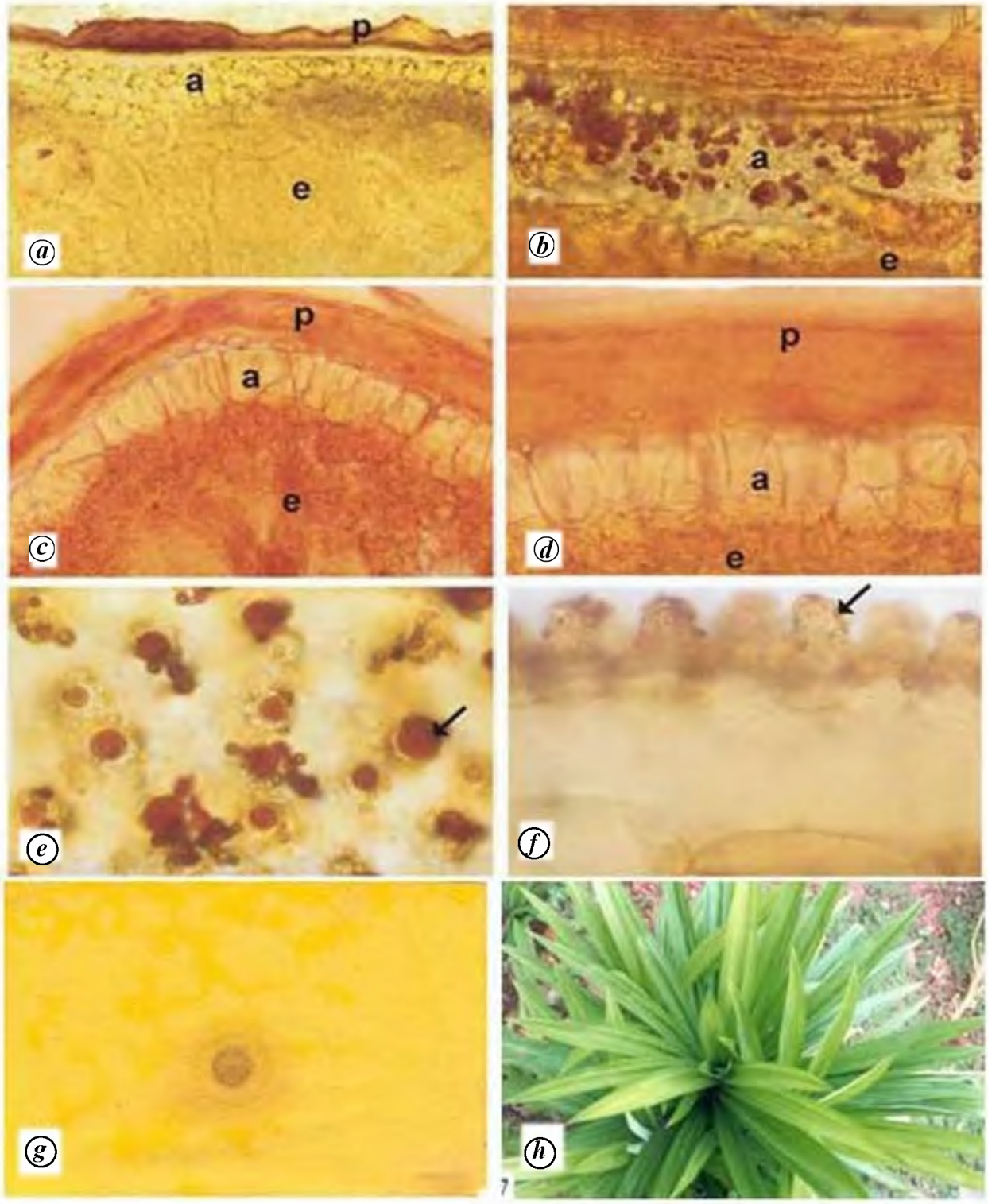


Figure 1. Histochemical localization of 2-acetyl-1-pyrroline. ***a***, Transverse section of mature rice caryopsis of Tarori basmati showing reaction with 2,4-dinitrophenyl hydrazine in the aleurone, X 800. ***b***, Enlarged view of (***a***) showing distinct orange-red bodies in aleurone indicating the presence of 2AP, X 2000. ***c***, Transverse section of mature rice caryopsis of non-scented rice variety Jaya showing no reaction with 2,4-dinitrophenyl hydrazine in aleurone, X 800. ***d***, Enlarged view of (***c***) showing no reaction in aleurone, X 2000. ***e***, Surface view of epidermal papillae of *Pandanus amaryllifolius* Roxb. leaf showing distinct reaction for 2AP with 2,4-dinitrophenyl hydrazine (arrow), X 2000. ***f***, Transverse section of epidermal papillae of *P. amaryllifolius* Roxb. leaf showing distinct reaction for 2AP with 2,4-dinitrophenyl hydrazine (arrow), X 2000. ***g***, Confirmation of reaction on chromatograph paper using volatile extract of *P. amaryllifolius* Roxb. ***h***, *P. amaryllifolius* Roxb. plant growing in garden. a, Aleurone; e, Endosperm; p, Pericarp.

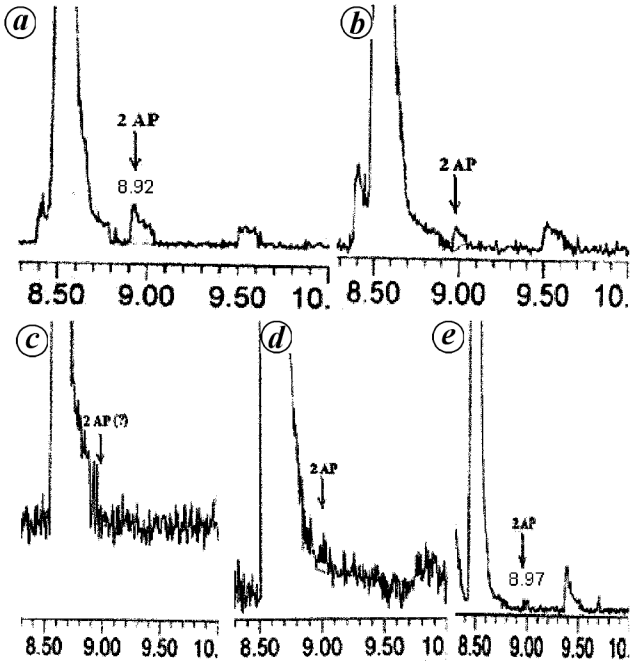
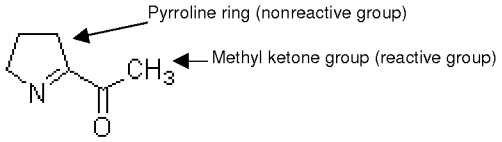


Figure 2. GC-MS analysis of aroma compounds. *a*, In marketed basmati rice (Kohinoor brand). *b*, In variety Pusa basmati grown during rainy season. *c*, In variety Pusa basmati grown during summer season. *d*, In local scented rice variety Ghansal. *e*, In non-scented rice variety Krishnahamsa. Note absence of 2AP peak (arrow).

For GC column, DB-5 (J&W Scientific, Folsom, CA) was used.

The results of histochemical localization of 2AP are shown in Figure 1 *a–g*. Figure 1 *a* shows the reaction in aleurone layer in the form of orange-red spots (magnified view in Figure 1 *b*). In the non-scented variety Jaya, no reaction was recorded (Figure 1 *c*, *d*). In addition, confirmative studies were carried out in *P. amaryllifolius*. The epidermal papillae showed distinct orange-red spots (Figure 1 *e*). The extracted volatile compounds also showed the typical colour reaction (Figure 1 *g*). Detection of aroma compounds in general and 2AP in particular, in scented rice varieties, has been attempted by many workers. Nagaraju *et al.*[6] developed a method to detect aroma that involves heating kernels/vegetative plant parts in water in closed vials and smelling it for aroma. Sood and Siddiq[7] developed a rapid technique for scent determination. They treated all the plant parts except roots with 1.7% potassium hydroxide for a short period and then the released aroma was inhaled. This was used worldwide as a basic technique for detection of aroma. In the present study, 2,4-dinitrophenyl hydrazine has been successfully used for the detection of 2AP. It is well established that 2,4-dinitrophenyl hydrazine reacts with methyl ketones to give an orange-red colour. The structure of 2AP shows that it has a non-reactive pyrroline ring and reactive methyl ketone group. The latter reacts with 2,4-dinitrophenyl hydrazine to give an orange-red coloured compound, 2-acetyl-phenyl hydrazone. The structure of 2AP and its reaction with 2,4-dinitrophenyl hydrazine is given below (Structure 1).



2-acetyl-1-pyrroline + 2,4-dinitrophenylhydrazine → 2-acetyl-phenyl hydrazone (orange-red colour)

Structure 1. 2-Acetyl-1-pyrroline.

In the present study, the histochemical reaction was also confirmed using *P. amaryllifolius* leaves (Figure 1 *e*, *f*). The leaves of *Pandanus* are rich in 2AP and it is most commonly used in cooking non-basmati rice to impart aroma of basmati rice. It has been reported that the aleurone layer contains higher quantities of 2AP than the endosperm[8,9]. In the present study, aleurone showed distinct reaction but 2AP was not visualized in the endosperm. Buttery *et al.*[8] have reported that in *P. amaryllifolius*, the 2AP content is ten times higher than in basmati rice. In the present study, histochemical staining reaction also clearly revealed the presence of 2AP in the leaves of *P. amaryllifolius* (Figure 1 *e*, *f*). The reaction was confirmed *in vitro* using spot test with the same reagent. In general,

Table 1. Quantification of 2-acetyl-1-pyrroline (2AP) in some scented rice varieties

Variety	2AP		TMP		Corr. area 2AP/TMP	Amount of 2AP in rice (g)	Average 2AP (ppm)
	Tr	Corr. area	Tr	Corr. area			
Marketed basmati B1	8.982	25961.00	11.674	303559.00	0.08552209	7.43 E-08	0.061
Marketed basmati B2	8.914	61235.00	11.596	802369.00	0.76317754	5.98 E-08	
Marketed basmati B3	8.917	64570.00	11.577	1100674.00	0.058664055	4.93 E-08	
Pusa basmati (rainy season-grown) R1	8.980	20115.80	11.730	323651.00	0.062150259	4.00 E-08	0.030
Pusa basmati (rainy season-grown) R2	8.907	10158.00	11.710	415302.00	0.024459309	1.51 E-08	
Pusa basmati (rainy season-grown) R3	8.959	21806.00	11.694	457615.00	0.04765141	3.67 E-08	
Pusa basmati (summer-grown) S1	–	–	11.793	82780.00	–	–	–
Pusa basmati (summer-grown) S2	–	–	11.702	118169.00	–	–	
Pusa basmati (summer-grown) S3	–	–	11.775	95672.00	–	–	
Ghansal G1	9.014	20914.00	11.749	454757.00	0.045989397	3.35 E-08	0.028
Ghansal G2	9.032	11007.00	11.697	673510.00	0.016342742	1.42 E-08	
Ghansal G3	9.032	46579.00	11.659	963571.00	0.048339977	3.79 E-08	
Krishnahansa K1	8.988	–	11.591	1794547	–	–	–
Krishnahansa K2	–	–	11.587	1898592.00	–	–	–
Krishnahansa K3	–	–	11.579	2146598.00	–	–	–

TMP, 2,4,6-trimethyl pyridine (internal standard).

2,4-dinitrophenyl hydrazine gives the same colour reaction with aldehydes and ketones. However, such compounds are present in trace quantities[3], beyond the level of localization as distinct reactions. Buttery *et al.*[9] have reported that the 2AP molecule is unstable in pure form. How it remains stable in plant systems is still unknown. They have also mentioned that the compound is released during cooking. Therefore, the colour reaction may be due to the release of 2AP when the sections/epidermal peels were incubated at higher temperatures.

Results of the biochemical analysis are depicted in Table 1 and Figure 2 *a–e*. The analysis showed that among the varieties studied, the marketed basmati rice showed maximum 2AP content (0.061 ppm). Pusa basmati grown in rainy season had 0.030 ppm 2AP followed by the local scented variety Ghansal (0.028 ppm). The non-scented variety Krishnahansa and Pusa basmati grown in summer season do not show the presence of 2AP. Buttery *et al.*[8] have reported 2AP content in some scented rice varieties. Their analysis of basmati 370 showed 0.06 ppm (in milled rice) and 0.17 ppm (in brown rice); Thai variety KDML had 0.07 ppm (in milled rice) and 0.20 ppm (in brown rice); Malakit Sungson 0.09 (in milled rice) and 0.20 ppm (in brown rice); Texas long grain <0.008 ppm; Calrose <0.006 ppm in milled rices. In another report Buttery *et al.*[10] reported that the 2AP content in cooked brown rice varieties such as Malakit Sungson was 760 ppb, Basmati 370 610 ppb, IR841761 560 ppb and Texas long-grain polished 6 ppb. The values of 2AP content obtained by us in the present study are comparable with these values. Results with Pusa basmati grown during different seasons revealed the temperature sensitivity of the 2AP molecule. Singh and Singh[11] reported that many indigenous non-basmati type scented rice varieties excel equally well as far as aroma and cooking qualities are concerned. 2AP content in the local scented non-basmati type variety Ghansal compares well with Pusa basmati, thus highlighting the importance of local scented rice varieties in both breeding programme and trade.

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