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Ornithine amino transferase (OAT) and δ^{1}-pyrroline 5-carboxylic acid synthetase (P5CS) in context to 2-Acetyl-1-Pyrroline (2-AP) in the seedlings of fragrant and non fragrant rice genotype(s)

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Abstract
2-Acetyl-1-Pyrroline is one of the potent flavor compounds in rice aroma. Studies were carried out to find the candidate genes/enzymes involved in the 2-AP biosynthesis from proline, glutamate and ornithine precursors. Ornithine Amino Transferase (OAT) and δ^{1}-Pyrroline 5-Carboxylic Acid Synthatase (P5CS) genes/enzymes were selected as the candidate genes/enzymes for the study in three rice genotypes Kalanamak 3119-2, Dehradun Basmati-3020 and Pant Dhan-4 (control). The specific enzyme activity pattern of OAT and P5CS was observed as Dehradun Basmati > Kalanamak > Pant Dhan-4. There was positive correlation between OAT and P5CS enzyme activity relative to 2-AP content. Genomic DNA of the three genotype(s) was subjected to PCR amplification using the gene specific primers of OAT and P5CS genes. Single band size of 604 bp was detected on the gel with OAT specific primers, while P5CS primers produced a 1671 bp band in all the three genotype(s). An additional band was also detected, whose size was slightly more than 604 bp when Dehradun Basmati-3020 genomic DNA was amplified using the OAT gene specific primers. Presence of two bands indicates that the genotype Dehradun Basmati-3020 may be containing more than one form of gene.

Keywords: ornithine amino transferase (OAT), δ^{1}-Pyrroline 5- carboxylic acid synthatase (P5CS) and 2-Acetyl-1-pyrroline (2-AP)

Introduction
Rice is the largest cereal crop in the world and it provides food for nearly half of the human race (Bose and Krishnamurthy, 1971). Nearly 85% of rice produced worldwide is used for human consumption, which is far higher than wheat (60%) and maize (25%) [1]. The global production of rice is estimated to be of the order of about 450 million tons per annum, which is cultivated in an area of about 145 million hectares. Aromatic rice has occupied a prime position in society for its aroma, milling, cooking and eating qualities and has been considered auspicious [2]. Among aromatic rice, the Basmati type is accepted as the best scented, longest and slenderest rice in the world. Consumers have become more quality conscious about the rice they consume. Modern biotechnological approaches for aroma enhancement offers advantages of no dependency over the environmental cues, which exert considerable influence on biosynthesis of valuable secondary metabolites [3]. Fragment rice is characterized by their unique popcorn like aroma that is attributed mainly to 2-AP [4-5]. 2-Acetyl-1-pyrroline had been proposed as a character impact aroma in rice at an extremely low odor threshold of 0.02ng/L [6-7]. 2-AP was identified for the first time in 1986 among food flavors as a constituent of cooked rice [8-9]. 2-AP is reported to be formed by the non-enzymatic browning reaction between reducing sugars and free amino acids, such as proline, ornithine and glutamate [10]. Proline is known to accumulate naturally in roots, leaves and grain of rice plants subjected to salinity stress [11]. Most plants appear to possess two Beatine aldehyde dehydrogenase (BADH 1 and BADH 2) genes and both of them have shown to play a role in drought and salinity stress responses in cereals [12-13]. The recessive gene *fgr* on chromosome 8 may encode a protein that either catalyses the formation or the removal of 2-AP. Fragrance is a recessive trait suggesting that a loss of function of an enzyme consuming a precursor of 2-AP is involved [14].

The genetic basis of fragrance has been studied by various workers and is reported to be controlled by one or two or three dominant and recessive genes [15]. It has been reported that proline, glutamate and ornithine are important precursor for 2-AP in rice seedling and callus [16]. Plants are known to show foliar accumulation of proline; defining the enzyme P5CS as a determinant in the foliar accumulation of this amino acid during salt stress [17]. Proline, glutamate and ornithine are the most probable precursors for 2-AP biosynthesis [18].

Our investigation is based on the production of 2-AP in aromatic Dehradun Basmati-3020, Kalanamak 3119-2 rice and non-aromatic variety Pant Dhan-4 rice. Precursor like compounds such as proline, glutamate and ornithine, related to 2-AP production pathway were tested, to study their effect on fragrance and their role as probable precursors for 2-AP biosynthesis. Further, Ornithine Amino Transferase (OAT) and δ^{1}-Pyrroline 5-Carboxylic Acid Synthatase (P5CS) assays were performed in different fragrant and non-fragrant rice genotype(s), so that these parameters could be correlated to understand the 2-AP metabolism.

Materials and Methods

Plant materials

Two rice cultivars of indigenous aromatic rice (*Oryza sativa* L.) and one non aromatic variety from different provinces of India were evaluated in this study. All the varieties were collected from Crop Research Centre of G.B.P.U.A.T., Pantnagar, India. Basmati rice variety selected was (Dehradun Basmati-3020), while non-basmati aromatic variety (Kalanamak 3119-2) and non aromatic (Pant Dhan-4) served as control in this study.

Isolation of enzymes (P5CS and OAT)

P5CS was isolated from the seedlings using 1 ml of extraction buffer containing 50mM Tris-HCl (pH 7.4) containing 7mM $MgCl_2$, 0.6M KCl, 3mM EDTA, 1mM DTT and 5% insoluble PVP [19]. For extraction of enzyme OAT, leaf tissues were homogenized with 100mM potassium phosphate buffer (pH 7.4), including 1mM pyridoxal-5-phosphate, 1 mM EDTA, and 10 mM β-ME in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 rpm for 20 min and the resulting supernatant was used for determination of the OAT activity. The whole extraction procedure was carried out at $4^{O}C$ [20].

Enzyme assay for P5CS and OAT

P5CS activity was assayed using a final reaction mixture with 0.5 ml volume (pH 7.0) containing 50 mM, L-glutamate 20 mM $MgCl_2$, 10mM ATP, 100mM dioxomate-HCl, 50 mM Tris and 0.5ml enzyme extract. After 5 min of incubation at $37^{0}C$, the reaction was stopped by addition of 0.5 ml of the stop buffer (2.5% $FeCl_3$ + 6% TCA) [21]. OAT activity was assayed in a final volume of 1 ml with the incubation medium (pH 8) containing 50mM ornithine, 20mM α-ketoglutarate, 1mM pyridoxal 5-phosphate and 100mM potassium phosphate buffer. Reaction was initiated by adding α-ketoglutarate. The incubation was carried out at 37 ^{O}C for 30 min. The reaction was stopped by adding 0.5 mL trichloroacetic acid (10%) and color was developed by incubating the reaction mixture with 0.5 mL o-aminobenzaldehyde (0.5%) in ethanol (95%) for 1 h. After centrifugation at 12,000 rpm for 10 min, the clear supernatant was taken to measure the optical density at 440 nm. One unit of OAT activity is defined as an increase of 1 A_{440} per h. OAT activity was expressed on the basis of g initial fresh weight [20] (Vogel and Kopac, 1996). Protein content was measured following Lowry method with BSA as a standard [22].

Precursor study on 2-AP formation in rice seedlings

Briefly, apical 5 cm segment excised from 14-days old seedlings were used for the precursor study. A group of 20 segments of excised rice seedlings (14 days old) were floated in 20 ml amino acid solution (pH 5.5) inside a Petri dish containing 500 mM proline, 500 mM ornithine and 500 mM glutamate separately, while distilled water served as a control. Incubation of seedlings was carried out at 27ºC in darkness for 8 hrs, after which segments were removed and rinsed with distilled water and 2-AP was extracted from samples [16] (Yoshihashi *et al.,* 2004).

Extraction of 2-AP from rice samples

Briefly, after an incubation of 8 hrs, segments of rice seedlings were crushed in appropriate amount of ethanol further incubated at room temperature for 2 hrs before proceeding for extraction. Extraction of 2-AP was performed in a separating funnel using DCM and water [18].

Quantification of 2-AP

For quantification of 2-AP in rice samples, thin layer chromatography was performed [18]. Reagent preparation was as per protocol reported previously [18]. 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 color. The structure of 2-AP shows that it has a non-reactive pyroline ring and reactive methyl ketone group. The latter reacts with 2, 4-dinitrophenyl hydrazine to give an orange-red colored compound, 2-acetyl-phenyl hydrazone.

Primer designing of OAT and P5CS genes

The sequences of rice OAT and P5CS genes were searched and downloaded from the NCBI (National Centre of Biotechnological Information) online database. Gene specific primers of these two sequences were designed using the online Primer3 tool integrated with the NCBI database. The generated primer sequences of the OAT gene were OAT-F 5'CTGGAGCTGAAGGAGTGGAAACAGC3' and OAT-R 5'GATGGCCAGGAACCAATGGG3' and for the P5CS gene, P5CS-F 5'GCAATCTGAACCAAGGCATCAGG3' and P5CS-R 5'TTTAGCAGGACTGTTGGCACTGG3', respectively. Primer BLAST against the rice genome sequences predicted the primers of OAT gene to amplify a region of 604 bp, while the P5CS primers amplified a region of 1671 bp of their respective genes.

DNA extraction and PCR amplification

Genomic DNA from rice genotypes was isolated from leaf samples (20 mg each) of one month-old plants raised in net-house using CTAB method [23]. The DNA was spooled out, washed twice with 70% ethanol and dissolved in TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) containing 25 mg/ml RNase-A and incubated at 37ºC for 30 min. Further, it was extracted with chloroform–isoamyl alcohol (24:1v/v), re-precipitated, dissolved in TE buffer and stored at $-20^{0}C$. Each PCR 25 µl reaction mix was composed of 1 × PCR buffer, 100 µM dNTPs, 0.4 µM of each primer, 1.2 mM $MgCl_2$, 1 unit Taq DNA polymerase and 40-60 ng template DNA. PCR amplification was performed with initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for

1 min, 72 °C for 1 min and final extension at 72 °C for 7 min before cooling down to 4 °C. Amplification products were stored at -20 °C till further use. PCR products were analyzed by 1% agarose gel electrophoresis using 100 bp DNA ladder (Fermentas).

Results and Discussion

Enzyme activity of OAT and P5CS

In higher plants, δ^1-pyrroline carboxylic acid can be synthesized via three enzymes namely; OAT, PRODH and P5CS. To examine the relationship between endogenous levels of δ^1-pyrroline 5-carboxylic acid and the activities of its synthesizing enzymes, i.e., the activities of OAT and P5CS enzymes were examined in seedlings of three rice varieties. Enzyme OAT is helpful in synthesis of proline from ornithine [24]. Proline can be synthesized either from glutamate or ornithine by a single bi-functional enzyme, P5CS /P5CR [21]. As shown in [Fig-1], OAT activity was higher in aromatic rice variety Basmati-3020 as compared to aromatic non-Basmati Kalanamak 3119-2 and control Pant Dhan-4. Similarly, [Fig-2] revealed that enzyme activity of P5CS was highest in rice variety Dehradun Basmati 3020 as compared to Kalanamak 3119-2 and control Pant Dhan-4. Significant differences ($P<0.05$) were seen among the different rice genotypes.

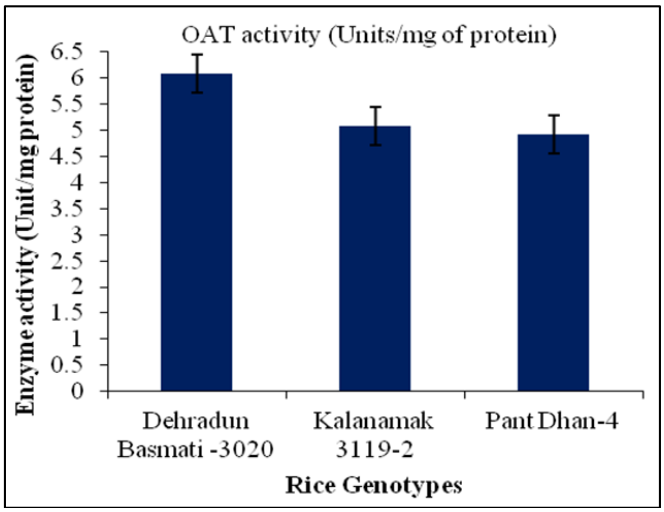


Fig 1: OAT enzyme activity/mg of protein in rice genotypes Dehradun Basmati-3020, Kalanamak 3119-2 and Pant Dhan-4

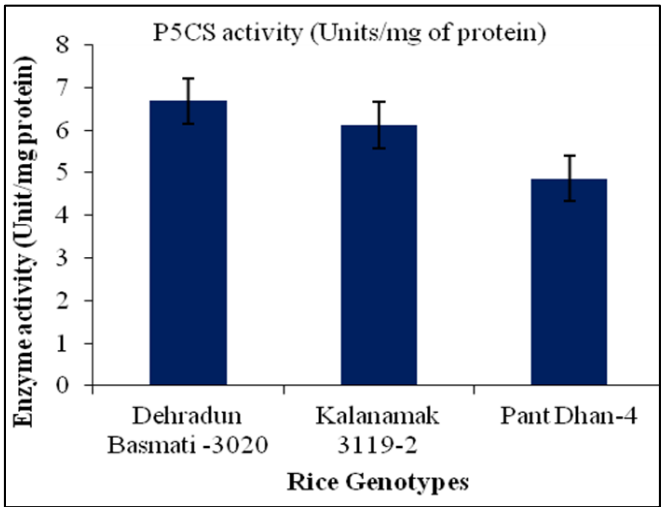


Fig 2: P5CS activity/mg of protein in rice genotypes Dehradun Basmati-3020, Kalanamak 3119-2 and Pant Dhan-4.

Our results show positive correlation between the activity of P5CS and 2-AP. There was positive correlation, between specific enzyme activities of OAT ($r = 0.60$) and P5CS ($r = 0.84$) relative to 2-AP concentration. Rice plants are known to enhance proline accumulation by increasing P5CS activity to alleviate the stress. A study revealed that 6.5% δ^1-pyrroline 5-carboxylic acid is automatically converted into 2-AP in the presence of trioses at room temperature, suggesting δ^1-pyrroline carboxylic acid to be precursor of 2-AP and that excess δ^1-pyrroline carboxylic acid is converted into 2-AP in order to decrease the toxicity of the highly oxidative intermediate compound methylglyoxal [25]. Similarly, it was demonstrated that in aromatic rice, increase in δ-^{1}pyrroline 5-carboxylic acid content has positive correlation with increase in activities of P5CS and OAT. The P5CS and OAT enzymes were found to be involved in the formation of proline and ultimately, 2-AP biosynthesis. Further, specific enzyme activity of acid δ^1-pyrroline 5-carboxylic synthesizing enzymes including δ^1-pyrolline 5-carboxylic acid synthetase (P5CS) and ornithine amino transferase (OAT) increased significantly in these aromatic rice varieties [26]. Another study has suggested that increase in 2-AP may be due to the higher level of δ^1-pyrroline 5-carboxylic acid and methylglyoxal accumulation in aromatic rice variety and that the level of δ^1-pyrroline 5-carboxylic acid was directly related with the activities of OAT and P5CS enzymes [25].

Precursor feeding

Amino acids related to proline and glutamate inter conversion pathway (proline, glutamate, ornithine) were used as probable precursors of 2-AP. Amount of 2-AP was measured by standard curve of 2-AP. Results obtained after thin layer chromatography (TLC) experiment revealed that the amount of 2-AP was maximum in treatments with glutamate as precursor (Dehradun Basmati-3010 (0.9µg)) > proline precursor (Dehradun Basmati-3010 (0.7µg)) > ornithine precursor (Dehradun Basmati-3010 (0.5µg)) as shown in Fig 3, 4 and 5.

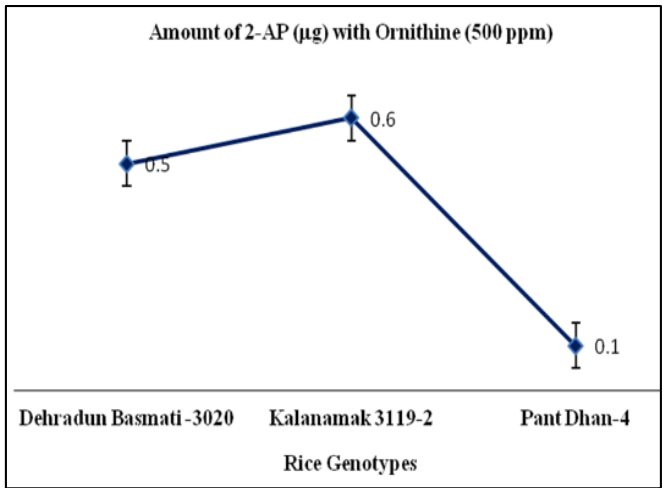


Fig 3: Amount of 2-AP in presence of ornithine in rice genotypes Dehradun Basmati-3020, Kalanamak 3119-2 and Pant Dhan-4.

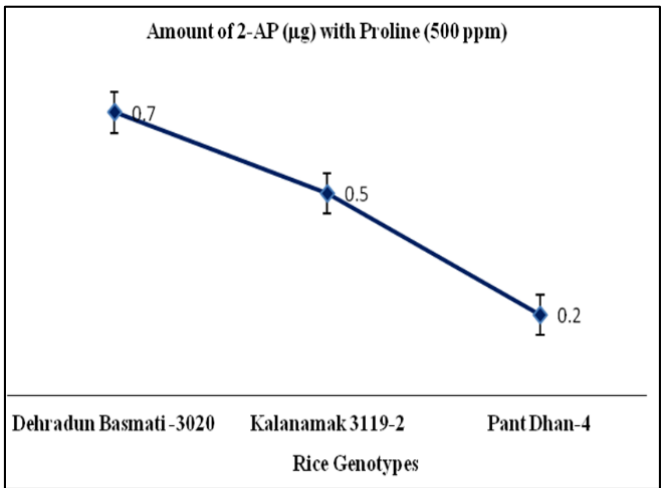


Fig 4: Amount of 2-AP in presence of proline in rice genotypes Dehradun Basmati-3020, Kalanamak 3119-2 and Pant Dhan-4.

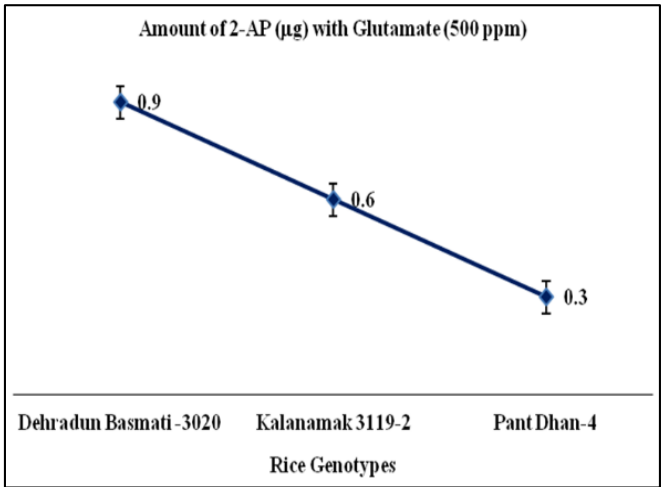


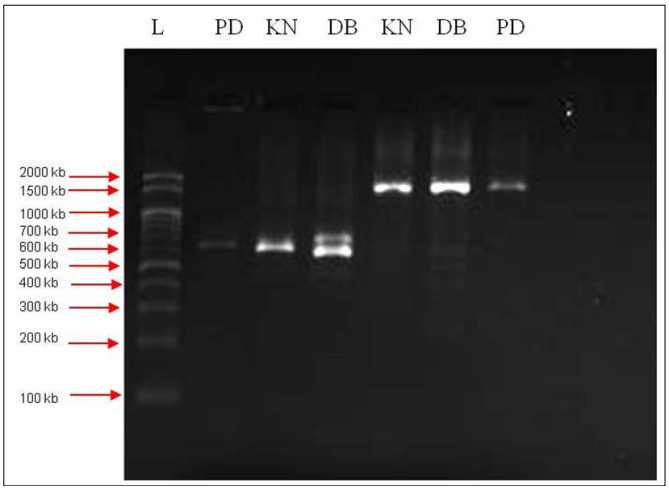
Fig 5: Amount of 2-AP in presence of glutamate in three rice genotypes Dehradun Basmati-3020, Kalanamak 3119-2 and Pant Dhan-4.

These results also indicate that L-glutamate was related to 2-AP formation in rice seedlings and that the formation of 2-AP was inherent in aromatic rice varieties tested (Dehradun Basmati-3020 and Kalanamak 3119-2). The results of precursor study clearly reveal glutamate to be the leading precursor than ornithine and proline in the production of 2-AP. Proline has been well known to accumulate in response to plant stress [27-28]. In higher plants under osmotic stress, proline is accumulated through stimulation of its *de novo* synthesis together with repression of its catabolism [24]. Proline can be synthesized from either glutamate or ornithine as its precursor. The first two steps of proline biosynthesis from glutamate are catalyzed by single bi-functional enzyme P5CS/ P5CR [21].

Present investigation shows that 2-AP content was higher in the seedlings of three rice cultivars that were incubated with glutamic acid for 8 hrs as compare to ornithine and proline treatments. It is well documented that glutamic acid is the common biosynthetic precursor of proline and ornithine. The catabolism of ornithine proceeds via 4-aminobutanal, which may catalyze to 1-pyrroline and 1-pyrroline has a well defined role in 2-AP biosynthesis [29]. A study concluded that proline was the most effective amino acid in increasing 2-AP in jasmine rice. Ornithine and glutamic acid increase 2-AP concentration at lesser levels, when a mixture of proline, ornithine and glutamic acid were added to Thai Jasmine rice and Khao Dwak 105 seedlings [30]. Our precursor feeding experiments results revealed higher 2-AP levels when seedlings were feed with glutamate as compared to proline and ornithine treatments, suggesting that glutamate was the main precursor for 2-AP biosynthesis. In rice, it has been demonstrated that glutamate is converted by bi-functional enzyme P5CS to glutamic γ-semialdehyde (GSA) which catalyses spontaneously to δ^1-pyrroline 5-carboxylic acid [25]. δ^1-pyrroline 5-carboxylic acid is then reduced into proline by P5CR. Two P5CS genes P5CS-1 and P5CS-2 have been identified in rice [31]. During osmotic stress such as drought and high salt level, proline is biosynthesized via intermediate δ^1-pyrroline carboxylic acid by glutamate and ornithine pathways. In our investigation, glutamic acid emerged as prominent precursor for proline synthesis and ultimately for 2-AP biosynthesis, as amount of 2-AP was measured to be highest when seedling tissue samples of Dehradun Basmati-3020 were incubated in glutamate. This suggests that δ^1-pyrroline 5-carboxylic acid derived from glutamic acid by P5CS may be reacting directly with methylglyoxal and leading to formation of 2-AP.

PCR amplification of OAT and P5CS genes

Genomic DNA of the three varieties was subjected to PCR amplification using the gene specific primers of OAT and P5CS genes Fig. 6. Gene specific PCR primers were used to detect and amplify OAT and P5CS genes in the genomes of the three varieties under study. Using the OAT gene specific primers, in all the three varieties a single band size of 604 bp was detected on 1% agarose gel, while P5CS primers produced a single band of 1671 bp size in all the three varieties. However, an additional band was also detected when Dehradun Basmati-3020 genomic DNA was amplified using the OAT gene specific primer, whose size was slightly more than 604 bp. This holds some significance because rest of the two varieties showed a single band after amplification. Presence of two bands indicates that the variety Dehradun Basmati-3020 may be containing more than one gene in the form of an isozyme which is involved in biosynthetic pathway of 2-AP.



L-1: Ladder, L-2: Pant dhan, L-3: Kalanamak,
L-4: Dehradun Basmati (with OAT gene), L-5: Kalanamak,
L-6: Dehradun Basmati, L-7: Pant Dhan (with P5CS gene)

Fig 6

Conclusion

In present study it was analyzed that OAT activity was higher in aromatic rice variety Basmati-3020 as compared to

aromatic non-Basmati Kalanamak 3119-2 and control Pant Dhan-4. Similarly enzyme activity of P5CS was highest in rice variety Dehradun Basmati 3020 as compared to Kalanamak 3119-2 and control Pant Dhan-4. Significant differences ($P<0.05$) were seen among the different rice genotypes. Results show positive correlation between the activity of P5CS and 2-AP. There was positive correlation, between specific enzyme activities of OAT and P5CS relative to 2-AP concentration. Rice plants are known to enhance proline accumulation by increasing P5CS activity to alleviate the stress. After thin layer chromatography (TLC) experiment revealed that the amount of 2-AP was maximum in treatments with glutamate as precursor. These results also indicated that L-glutamate was related to 2-AP formation in rice seedlings and that the formation of 2-AP was inherent in aromatic rice varieties. The results of precursor study clearly reveal glutamate to be the leading precursor than ornithine and proline in the production of 2-AP.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. This manuscript has not been published and is not going to be considered for publication elsewhere. The authors certify that neither the manuscript nor its main contents have already been published or submitted for publication in another journal.

Author's Contributions

All the authors contributed equally to the writing of this paper. They were also involved in the overall work of experiments

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