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Paclobutrazol persistence dynamics in mango (*Mangifera indica*) orchard soil using HPLC and LC-MS/MS analysis

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ABSTRACT

Paclobutrazol, a plant growth regulator, is used commercially in soil application to counter the alternate bearing habit of mango (*Mangifera indica* L.) all over the country. The experiments were conducted during 2020–21 and 2021–22 at ICAR-Central Institute for Subtropical Horticulture, Lucknow, Uttar Pradesh to study the persistence of paclobutrazol in soil and its subsequent uptake in mango fruits using HPLC and validated by LC-MS/MS after its application to tree basin soil of mango cv. Dasheri @2, 4, 6, 8 and 10 g a.i./tree. The residues of paclobutrazol dissipated in soil from its initial values of 10.33, 17.25, 25.71, 50.80, and 62.36 µg/g soil at 0 days to 0.14, 0.30, 0.47, 1.32 and 2.38 µg/g soil after 300 days of application. The dissipation rate in soil followed first-order kinetics with half-life values ranging between 57.75 and 77.00 days. Residues of paclobutrazol were not detected in fully mature fruits harvested after 85 days of fruit set. LC-MS/MS analysis confirmed that the sample peaks corresponded to paclobutrazol residues in mango fruit and soil. Thus, HPLC and LC-MS/MS analysis clearly showed that the paclobutrazol persisted in the soil for longer, but the mature fruits harvested from treated trees were free from its residue even at higher doses and safe for consumption. The response of paclobutrazol to the quality of fruits in terms of phenolic content was also assessed. The paclobutrazol was more responsive in altering phenolic acid composition than the control.

Keywords: LC-MS/MS, Mango, Paclobutrazol, Residue, Soil

India's and the world's most valuable fruit crop is mango (*Mangifera indica* L.). Dasheri is India's most famous mango cultivar having low productivity due to biennial bearing. Paclobutrazol (2RS, 3RS)-(4-dichlorophenyl)-4-dimethyl-2-(1,2,4-triazole) pentane-3-ol induces mango flowering in off years by blocking gibberellin biosynthesis (Burondkar and Gunjate 1993, Singh *et al.* 2005). It can be sprayed on mango tree leaves, soaked in soil, or painted on their trunks, but soil treatment in the collar or root zone worked best (Pujari *et al.* 1999). According to research, paclobutrazol must be applied annually to boost mango harvests (Yadava and Singh 1998). Paclobutrazol also affects soil microbial activity and nutrients (Silva *et al.* 2003, Singh and Bhattacherjee 2005, Kotur 2012, Kumar *et al.* 2021b). Paclobutrazol's high persistence and low mobility reduce its environmental contamination risk but cannot be eliminated. In heavily applied areas, its long soil persistence can pollute the environment. Paclobutrazol remains active in soil for a long time, and its half-life varied by soil type and weather (Costa *et al.* 2012). Paclobutrazol has high soil persistence, but mature mango fruits are residue-free (Sha1rma and Awasthi 2005). Paclobutrazol was mostly translocated by the xylem (through the stem) and accumulated in leaves (Orozco-Melendez *et al.* 2022). Paclobutrazol residues in leaves and ripe fruits were trace (<MRL) at lower concentrations but increased significantly with higher concentrations (Jorjong *et al.* 2022). GLC (Wang *et al.* 1986) and HPLC (Parvathamma *et al.* 2012) can analyse paclobutrazol persistence in various matrices. Further LC-MS/MS can verify and validate paclobutrazol residue in soil and mature fruits. LC-/MS/MS is the preferred technology for chemical analysis due to its selectivity, sensitivity and adaptability to various sample matrices, and chemicals (Pareja *et al.* 2011).

This study examined paclobutrazol persistence in soil after soil drenching in bearing young Dasheri mango trees and its uptake in mango fruits to determine its translocation status and safety for human consumption. Phenols improve mango fruit quality, thus, the present article also examined paclobutrazol's response to phenolic compound changes in mature mango fruits.

MATERIALS AND METHODS

Application of paclobutrazol: The experiments were conducted during 2020–21 and 2021–22 at ICAR-Central

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Institute for Subtropical Horticulture, Lucknow, Uttar Pradesh at an orchard of 0.25 ha with a planting distance of 9 m × 6 m and 18-year-old, 48 mango (cv. Dasheri) trees were taken. Mango season averaged temperature ranges between 12.5–32.5°C with 85–90% relative humidity and 765 mm of annual precipitation. In the 4th week of September, a 15 cm deep, 30 cm wide furrow was dug at a radial diameter of 1.0–1.5 m from the tree trunk and treated with paclobutrazol @2.0 (T_2); 4.0 (T_3); 6.0 (T_4); 8.0 (T_5); and 10.0 (T_6) g a.i./tree as Cultar® 23 SC. Untreated trees were used as control (T_1). Three identical trees per treatment were kept. For uniform distribution, 15 litres of water were poured into the soil above furrow with the required paclobutrazol dosage. A mechanical soil auger randomly sampled soil from 0–15 cm depth 1 h after application (the first day), 30, 60, 90, 120, 150, 240, and 300 days after treatment. Paclobutrazol residues were measured in the fruit's climacteric phase 255 days after treatment.

Long-term residual impact on fruit yield: In the same orchard, paclobutrazol residue's long-term effects on mango cv. Dasheri assessed on flowering and fruiting. First-year paclobutrazol applications were 2, 4, 6, 8, and 10 g a.i./tree. To study paclobutrazol's long-term effects on mango production, the plants were split into three sets in second year. One set of plants received the full dose from previous years, the second half, and the third none. Paclobutrazol-free trees were controls. The residual soil level and harvest yield were measured.

Recovery of paclobutrazol in soil and fruit: A 1000/mg paclobutrazol stock solution was made by dissolving a technical standard in HPLC-grade methanol. To test the analytical approach, HPLC spiked paclobutrazol in mango fruit and soil at 1.0, 5.0, and 10.0 ppm, while LC-MS/MS measured it at 0.01, 0.05, and 1.0 ppm. Residue data were statistically analyzed to determine residual half-life (DT50 in days) (Hoskins 1961). The orchard soil was loam to fine sandy loam (1250 ppm), neutral, and high in calcium.

Extraction of paclobutrazol residues from soil and fruit: Paclobutrazol residues were extracted from soil using the technique reported by Bhattacherjee and Singh (2002) and its residues in soil and fruits were analyzed as per the method described by Bhattacherjee and Singh (2015).

Degradation kinetics of paclobutrazol: The degradation kinetics of paclobutrazol in orchard soil was calculated as per the method described by Kumar *et al.* (2021a).

Analysis of paclobutrazol in mango fruits and soil by LC-MS/MS: For LC-MS/MS analysis, mango fruit paclobutrazol residue was extracted using ethyl acetate and centrifugation at 15,000 rpm with the QuEChERS method. In soil, the residue were extracted using acetonitrile and centrifugation at 3000 rpm. The filtered final volume was analyzed using LC-MS/MS like fruit samples.

Sampling/Chemicals: For residue quantification using LC-MS/MS, the soil samples from all paclobutrazol concentrations were taken at 180 days following its application. Whereas the residual analyses for fruit samples were taken from control, 4.0, 6.0, and 10.0 g a.i./tree. Paclobutrazol, the analyses certified reference material (CRM), was obtained from Dr. Ehrenstorfer GmbH in Germany and was 99.0% pure.

Analytical conditions of LC-MS/MS: Electrospray ionisation source (ESI) in positive ionization mode operated the LC-MS/MS (Waters UPLC linked with AB Sciex-3200) with Chromolith RP-18e column (100 mm × 4.6 mm). The gradient of solvents A and B in the LC mobile phase ran for 20.0 min at 0.45 ml/min (5 mM ammonium formate-water and methanol, respectively). Injecting 10 µl at 8.99±0.1 min eluted the peak. Paclobutrazol residues were analyzed using multi-reaction monitoring (MRM). MRM transitions last 20 msec. The ion source stayed at 55ºC.

Measurements of phenolic content: The results presented pooled data from 10 uniformly ripened and blemish-free fruits from paclobutrazol-treated (4.0 and 8.0 g a.i.) and untreated trees in 2020–21 and 2021–22. For phenolic compound estimation, triplicate homogenized fruit samples were collected. HPLC estimated phenolic compounds (HPLC). Sigma-Aldrich India Mumbai supplied phenolic reference standards. As described earlier, gallic acid, chlorogenic acid, catechins, epicatechin, and caffeic acid were extracted (Bhattacherjee *et al.* 2020). Agri Stat software analyzed all data and compared means at P=0.05.

RESULTS AND DISCUSSION

Recovery of paclobutrazol from soil and fruit/pulp: Paclobutrazol was recovered from the soil in a range of 90.48–92.52% using HPLC. Paclobutrazol recovery from mango pulp ranged from 80.78–82.59% after fortification, and equivalent LC-MS/MS values for soil and mango fruit were 89.91–99.36 and 96.32–99.125%, respectively.

Residues of paclobutrazol in soil: HPLC data showed that after 150 days of application, it dissipated to 1.55, 3.73, 4.58, 8.34, and 8.42/µg in soil from 10.32, 17.25, 25.71, 50.80, and 62.36 µg/g. After 300 days, 2.0, 4.0, 6.0, 8.0, and 10.0 g a.i. degraded to 0.14, 0.30, 0.47, 1.32, and 2.38 µg/g. Using 2.0 to 10.0 g a.i./tree, the computed residual half-life values ranged from 57.75–77.00 days (Table 1). Soil residue was absent from the control treatment. After 300 days in the soil, over 99% of residues from all concentrations were degraded. Paclobutrazol dissipation in soil followed pseudo-first order kinetics because it was faster up to 90 days and slower after 90 days (Fig. 1). After 180 days, LC-MS/MS determined the average residues in soil samples for dosages of 0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 g a.i./tree as BDL, 0.82, 1.11, 1.63, 3.98, and 5.66 g/g, respectively (Fig. 2). The residue level increased with paclobutrazol concentration, supporting HPLC data (Fig. 3A). LC-MS/MS showed that paclobutrazol residues were present. Paclobutrazol lasted 210 days in southern India's mango orchard soil (Shalini and Sharma 2006). Mango cultivars sometimes flowered from residue. In mango leaf and inflorescence studies (Bhattacherjee and Singh 2015), paclobutrazol residue was initially undetectable. However, paclobutrazol residues in mango trees after soil drenching, tree injection, root zone injection, or foliar spray need to be compared.

Table 1 Residues of paclobutrazol from different concentrations in mango cv. Dasheri orchard (2020–21)

Days after application	Residues (µg/g) ± SD*				
	T_2 (2.0 g a.i./tree)	T_3 (4.0 g a.i./tree)	T_4 (6.0 g a.i./tree)	T_5 (8.0 g a.i./tree)	T_6 (10.0 g a.i./tree)
0	10.33 ± 0.29	17.25 ± 0.61	25.71 ± 0.53	50.80 ± 0.76	62.36 ± 0.46
30	6.41 ± 0.	9.20 ± 0.25	10.71 ± 0.20	19.45 ± 1.82	33.04 ± 1.74
60	5.51 ± 0.20	8.09 ± 0.14	7.79 ± 0.07	9.10 ± 0.02	13.19 ± 1.39
90	4.23 ± 0.22	6.92 ± 0.23	6.71 ± 0.09	9.06 ± 0.26	10.48 ± 0.20
120	3.37 ± 0.08	6.42 ± 0.21	5.42 ± 0.06	9.04 ± 0.02	9.34 ± 0.10
150	1.55 ± 0.07	3.73 ± 0.01	4.58 ± 0.08	8.34 ± 0.06	8.42 ± 0.20
240	1.29 ± 0.03	2.41 ± 0.03	3.36 ± 0.09	4.57 ± 0.11	4.91 ± 0.05
300	0.14 ± 0.01	0.30 ± 0.02	0.47 ± 0.06	1.32 ± 0.33	2.38 ± 0.16
Dissipation (%)	98.64	98.26	98.17	97.40	96.18
Half-life (days)	57.75	63.00	69.30	77.00	77.00
Regression equation	$11.63e^{-0.012x}$	$17.97e^{-0.011x}$	$19.03e^{-0.01x}$	$28.52e^{-0.009x}$	$35.92e^{-0.009x}$
Regression coefficient	0.902	0.884	0.873	0.865	0.889

*SD, Standard deviation; T_1, Control (residues were not detected and values are not mentioned in the Table).

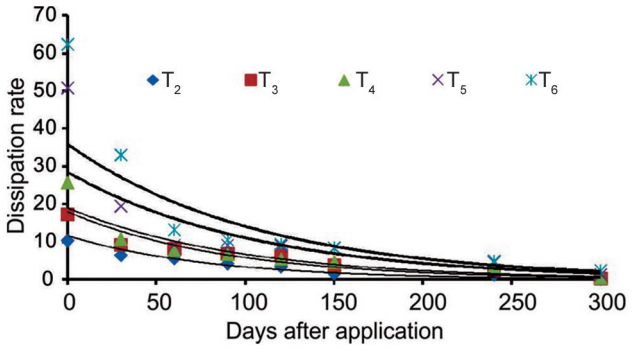

Fig. 1 First-order exponential dissipation of paclobutrazol residues (µg/g) day after treatment.
Axis X is the day after PBZ treatment, Y is dissipation rate. T_2, 2.0 g a.i./tree; T_3, 4.0 g a.i./tree; T_4, 6.0 g a.i./tree; T_5, 8.0 g a.i./tree; T_6, 10.0 g a.i./tree.

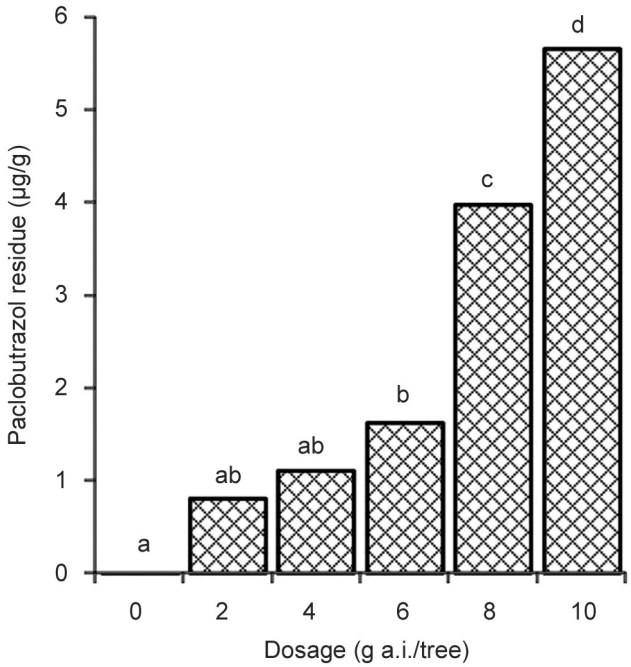

Fig. 2 Residue level of paclobutrazol in mango cv. Dasheri soil analysed through LC-MS/MS.

Long-term residual impact of paclobutrazol on fruit yield: The first year of paclobutrazol application (2020–21) showed that it increased fruit yield by 8.0 g a.i. (58.33 kg) and 10.0 g (55.83 kg)/tree. When the same dose was applied to the same trees in the second year, yield increased marginally at different concentrations (2.0–8.0 g a.i.). Compared to the control, 10 g a.i. yield increased significantly. Reducing the paclobutrazol dose to half in second year increased fruit yield compared to the meager control yield (5.75 kg/tree). In another set of experiments, when paclobutrazol was not applied to the previous year's treated trees, most treatments yielded very low, except for the trees treated with 10.0 g a.i. in the last year (2020–21). Thus, mango fruiting is not promoted by the low soil residue left after paclobutrazol was not given the following year. Based on this study, paclobutrazol in the first year and half dose in the second year can regulate mango cv. Dasheri fruiting. Paclobutrazol persists in mango orchards and promotes mango fruiting (Table 2 and Fig. 3B). Paclobutrazol was degraded to 0.93–3.28 µg/g at 240 days, less than the total dose. When the same amount was repeated in 2021–22, the residue level in the soil was marginally increased and dissipated in the range of 1.96–6.89 µg/g after 150 days (Table 2 and Fig. 3C). When paclobutrazol was not applied the following year, residue in the soil was only detected at 8.0 g a.i. (0.08–0.05 µg/g) and 10.0 g a.i. (0.10–0.04 µg/g).

Residues of paclobutrazol in mango fruit: HPLC estimates showed no paclobutrazol residue in mature fruit pulp at harvest (255 days after application) (Table 3). In all treatments, LC-MS/MS found no paclobutrazol

Table 2 Residues of paclobutrazol from different concentrations in mango cv. Dasheri orchard in second year (2021–22)

Treatment (g a.i./tree)	Residues (µg/g)				
	0 day	90 day	120 day	150 day	240 day
Residue level of paclobutrazol in second year (2021–22) applied full dose as of Ist year					
T_1, Control	-	-	-	-	-
T_2, 2.0	13.51 ± 0.57	5.66 ± 0.06	2.96 ± 0.18	2.20 ± 0.18	1.96 ± 0.02
T_3, 4.0	20.22 ± 2.64	8.27 ± 0.52	4.96 ± 0.53	3.03 ± 0.59	2.56 ± 0.36
T_4, 6.0	30.14 ± 0.61	8.11 ± 0.38	7.85 ± 0.49	4.99 ± 0.54	4.02 ± 0.26
T_5, 8.0	56.91 ± 2.18	10.58 ± 0.82	10.01 ± 0.22	7.02 ± 0.38	6.25 ± 0.45
T_6, 10.0	63.38 ± 1.04	12.33 ± 0.24	10.85 ± 0.58	7.69 ± 0.36	6.89 ± 0.62
Residue level of paclobutrazol in the second year (2021–2022) applied half dose of Ist year					
T_1, Control	-	-	-	-	-
T_2, 2.0	6.12 ± 0.19	2.32 ± 0.35	2.03 ± 0.10	1.62 ± 0.03	0.93 ± 0.23
T_3, 4.0	12.11 ± 0.45	4.07 ± 0.38	3.62 ± 0.22	2.55 ± 0.36	1.57 ± 0.28
T_4, 6.0	12.24 ± 0.39	5.25 ± 0.24	2.77 ± 0.34	2.60 ± 0.30	1.03 ± 0.03
T_5, 8.0	25.14 ± 0.60	5.68 ± 0.21	4.92 ± 0.44	3.14 ± 0.08	3.12 ± 0.09
T_6, 10.0	30.78 ± 0.53	6.98 ± 0.31	5.54 ± 0.27	3.58 ± 0.25	3.28 ± 0.21
Residue level of paclobutrazol second year (2021–22) (No treatment of 1st year)					
T_1, Control	-	-	-	-	-
T_2, 2.0	BDL	BDL	BDL	BDL	BDL
T_3, 4.0	BDL	BDL	BDL	BDL	BDL
T_4, 6.0	BDL	BDL	BDL	BDL	BDL
T_5, 8.0	0.08 ± 0.02	0.05 ± 0.03	BDL	BDL	BDL
T_6, 10.0	0.10 ± 0.01	0.09 ± 0.03	0.09 ± 0.03	0.08 ± 0.01	0.04 ± 0.01

BDL, Below detectable level.

residues in fruits. Its biodegradation in leaves, flowering shoots, or paclobutrazol lowered hydraulic conductivity, slowing the transfer of its residue to fruits, which may explain why ripe fruits have no residue. 'Tommy Atkins' fruits were 14C-paclobutrazol-free after soil treatment (Costa *et al.* 2012). Sharma *et al.* (2008) also found no paclobutrazol residues in Alphonso above 0.01 mg/kg, despite the number of applications in the Konkan region of Maharashtra over the past 10 years. HPLC and LC-MS/MS analysis showed that paclobutrazol remained in the soil longer, but mature fruits from treated trees were residue-free and safe to eat. Mango fruit had no paclobutrazol residue after 2 years of treatment (data not shown). The current study suggested that using paclobutrazol to prevent alternate bearing in mangoes at acceptable doses continuously may not leave harmful residues in mango fruits. However, paclobutrazol's long soil persistence may pollute areas where it is applied. Thus, paclobutrazol leaching and residue prevention near tree basins must be studied.

Table 3 Impact of paclobutrazol residue on fruit yield in mango cv. Dashehari

Treatment (g a.i./tree)	Fruit weight (kg)/tree			
	Full dose (2020–21)*	Full dose (2021–22)**	Half dose of (2021–22)***	Nil dose of the previous year (2021–22)****
T_2, 2.0	37.00	54.45	43.75	21.30
T_3, 4.0	42.33	56.50	23.75	24.10
T_4, 6.0	47.50	43.00	40.12	26.50
T_5, 8.0	58.33	54.10	65.50	12.50
T_6, 10.0	55.83	72.92	13.00	72.34
T_1, Control	36.66	17.25	5.75	11.50
CD (*P*=0.05)	2.75	1.84	5.98	9.56

*Full dose of PBZ during 2020–21; ** Full dose of PBZ (2021–22) in the previous year's (2020–21) treated trees; *** Half dose of PBZ (2021–22) in the previous year's (2020–21) treated trees; ****No treatment of PBZ in the previous year's (2020-21) treated tree.

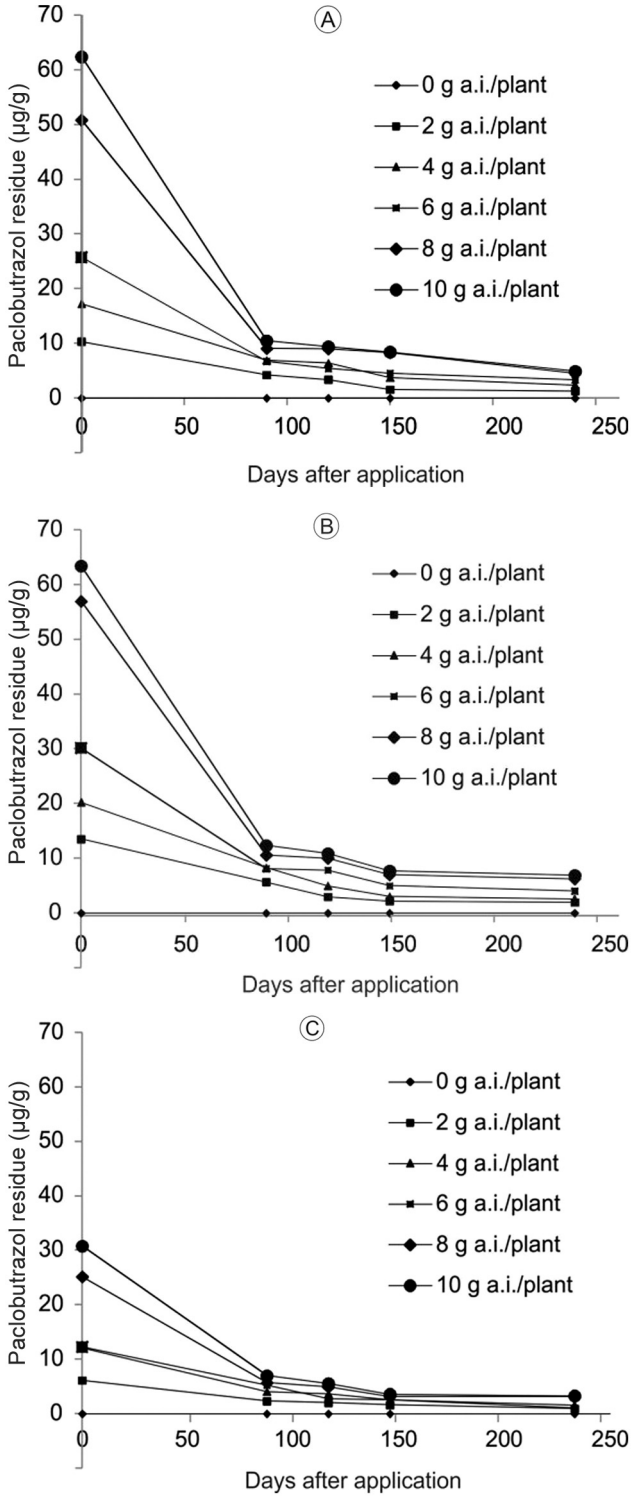

Fig. 3 Long-term impact of paclobutrazol on residual level in soil.
(A) PBZ residue in 1st year (2020–21) applied full dose; (B) PBZ residue in 2nd year (2021–22) applied full dose as of 1st year; (C) PBZ residue in 2nd year (2021–22) applied half dose of 1st year.

Response of paclobutrazol on changes in phenolic compounds in fruits of mango cv. Dasheri: The phenolic acid content of mango cv. Dasheri fruits treated with paclobutrazol exhibited significant variations compared to the control group. Gallic acid levels were 58.07 ± 0.89 µg/g in the control, 66.20 ± 5.00 µg/g with 4.0 g a.i./tree treatment, and 59.61 ± 3.12 µg/g with 8.0 g a.i./tree treatment. Catechin content was 67.92 ± 6.20 µg/g in the control, 133.99 ± 13.29 µg/g with 4.0 g a.i./tree treatment, and 121.69 ± 9.37 µg/g with 8.0 g a.i./tree treatment. Epicatechin levels were 80.65 ± 7.17 µg/g in the control, 183.93 ± 19.30 µg/g with 4.0 g a.i./tree treatment, and 198.31 ± 4.96 µg/g with 8.0 g a.i./tree treatment. Caffeic acid content was 7.30 ± 0.56 µg/g in the control, 19.76 ± 1.61 µg/g with 4.0 g a.i./tree treatment, and 23.48 ± 2.18 µg/g with 8.0 g a.i./tree treatment. These results indicate (Supplementary Table 1) that paclobutrazol application led to significant changes in phenolic acid levels, with higher doses generally resulting in higher phenolic acid content.

The study revealed that paclobutrazol application in mango cv. Dasheri trees significantly impacts its levels in fruits and soil residue. Recoveries of 90.48–92.52% from soil and 80.78–82.59% from fruit/pulp indicate its persistence. Residues in soil decline over time and pseudo-first order kinetics show its 99% degradation after 300 days. Fruit yield increases with paclobutrazol, even at reduced doses in subsequent years, indicating a lasting impact on fruit production. However, residue levels in mature fruit pulp at harvest are undetectable, ensuring fruit safety. Overall, paclobutrazol effectively regulates mango cv. Dasheri fruiting, with minimal impact on fruit quality and safety. Nonetheless, further research is necessary to assess its long-term environmental impact and develop sustainable management practices.

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