Aril browning in pomegranate (*Punica granatum* L.) is caused by the seed

Aril browning (AB) in pomegranate (*Punica granatum* L.) is a physiological disorder critically affecting fruit quality in some commercially important varieties such as cv. Ganesh and cv. Bhagwa. As the fruits affected by the disorder remain free from external symptoms, they cannot be separated out before being packed, thus posing serious problems in export trade. Consequently, the problem has assumed great significance and research efforts to identify the underlying mechanism of AB are in progress in many countries all over the world. In spite of extensive studies, the problem has remained an enigma till date.

AB is characterized by soft, light creamy, brown, dark blackish or brown and slightly flattened arils which are deformed and possessing an unpleasant odour when the fruit is cut open[1] (Figure 1). The disorder is accompanied by desiccation, wrinkling and development of internal spaces in the arils. Browning of the arils starts with a tiny dark dot on the seed, which later spreads to the entire aril and many arils exhibit a streaked appearance due to fine white lines radiating from the seeds. The affected fruits exhibit poor dessert quality and are unfit for consumption in the advanced stages of the disorder. In many cases, the intensity of the disorder in mature ripe fruits could be more than 50% causing severe loss of quality.

Extensive studies on AB have shown that the malady is influenced by diverse factors such as genetic background, pruning, growing season, fruit size, harvest date and variety[2], but they could not determine the causative factor. Biochemical studies also could not throw light on the possible origin of the malady[3,4]. A comprehensive analysis of the available literature on the subject revealed that all the past studies have focused attention exclusively on the changes occurring in the juice as a result of the disorder, but precious little on the seed. Interestingly, the pulpy aril in pomegranate is nothing but a fleshy outgrowth from the funiculus that surrounds the seed[5]. It is well established that the fruit growth is inseparably related to the activity of the seed[6]. As a result, any change in the seed metabolism is likely to impact fruit quality. Considering the pivotal role played by the seed in the growth and development of the fruit, a comparative study of the physiological and biochemical changes in both seed and juice of healthy (H) and AB-affected arils was carried out with the aim of identifying the causative factor responsible for initiating the phenomenon of AB.

Figure 1. Healthy and browned arils of pomegranate fruit.

Pomegranate fruits of cv. Bhagwa were collected from a farmer's orchard located in Sira, Tumkur District, Karnataka and brought to laboratory. Fruits for analysis were harvested at 90% maturity and ripened at room temperature (26 ± 2°C) and relative humidity (70 ± 5%) for 4–10 days. Ripe fruits were cut open and the AB-affected arils were separated out from each fruit. The seed and juice of healthy and browned arils from each fruit were separated and analysed. To study the causative role of the seed in AB formation, preharvest treatment of developing fruits at 50% and 80% maturity was carried out. For this study, fruits were dip-treated with the plant growth regulators (PGRs), gibberellic acid (GA_3) (growth promoter) @ 500 ppm and paclobutrazol (PBZ) (anti-gibberellin) @ 1000 ppm mixed with a non-ionic adjuvant, APSA-80 @ 0.03% for 1 min. Water with adjuvant acted as control. Fruits were harvested on the 126th day when they had attained 90% maturity. Data on extent and rate of seed germination were collected on 400 seeds each of H and AB arils by recording the number of days taken by each seed from sowing to the emergence of sprout.

The incidence of AB (AB%) was determined as percentage of the ratio of the number of browned arils to the total number of arils present. Moisture content of juicy pulp and seed samples was estimated gravimetrically. Pulp and seed tissues from healthy and AB-affected arils were analysed for reducing sugars, total sugars and starch using the dinitrosalicylic acid method[7]. Total soluble solids (TSS), titratable acidity (TA), pH and total phenols were determined following the method of Tehranifar *et al.*[8]. Protein was estimated using folin phenol reagent[9]. Total anthocyanins in the juice was determined by recording the absorbance at 540 nm (ref. 10). Ascorbic acid was estimated titrimetrically[11]. Amylase activity was assayed according to Bernfeld[12] and polyphenol oxidase (PPO) activity was determined following Esterbaner[13]. Seed viability was measured by assay of total dehydrogenase (TDH) activity using TTC test[14]. Plant growth hormones, GA_3 and indolyl 3-acetic acid (IAA) were analysed by HPLC, as described by Kelen *et al.*[15]. Ten fruits were sampled from each treatment and replicated for biochemical studies. Experimental data were subjected to ANOVA adapting the Fisher's analysis of variance technique[16] and mean values were tested for significance using Student's *t*-test.

During the studies, significant differences in the viability of seeds from H and AB-affected arils were observed. Studies were carried out to understand the role of seed viability in the development of AB in pomegranate. Table 1 reveals that the moisture content in H seeds was higher (47.89%) than that in the AB-affected seeds (44.22%). Percentage germination of seeds from AB-affected fruits was 70.25 as against 91.00 in seeds from healthy fruits. The mean number of days taken for germination was also higher in AB-affected fruits (37.55 days) as against 26.27 days in healthy fruits (Table 1). Supporting the above observations, TDH activity was higher in H seeds compared to AB seeds. Previous reports have established a direct correlation between seed viability and TDH activity[17]. Analysis of data showed that the reduced moisture content of seeds in AB led to reduced viability of seeds in AB-affected arils compared to H arils. Biochemical analysis of seeds showed that the activity of amylase was

Table 1. Biochemical parameters in healthy and aril browning affected seeds of pomegranate

Parameters	Healthy	Aril-browned	CD (P = 0.01)
Moisture (%)	47.89	44.22	1.8819
Total sugar (g/100 g fr wt)	1.040	3.025	0.2959
Reducing sugar (g/100 g fr wt)	0.967	2.112	0.3017
Protein (mg/100 g fr wt)	0.82	0.61	0.0838
Starch (g/100 g fr wt)	10.88	18.93	1.5179
TDH activity (ΔA_{485}/g)	2.805	1.383	0.4068
Amylase activity (mg maltose/g/h)	6.982	1.772	0.3847
GA_3 (µg/g fr wt)	0.841	0.646	0.0429
IAA (µg/g fr wt)	3.576	2.578	0.252
Germination (%)	91.00	70.25	3.1198
Rate of germination (no. of days)	26.27	37.55	2.4026

Table 2. Biochemical parameters in the juice of healthy and AB-affected pomegranate arils

Parameters	Healthy	Aril-browned	CD (P = 0.01)
Moisture (%)	83.67	81.13	1.1853
Total sugar (g/100 g fr wt)	8.44	6.67	0.7303
Reducing sugar (g/100 g fr wt)	5.34	4.18	0.4082
Total soluble solids (° Brix)	14.40	16.30	0.5455
Starch (g/100 g fr wt)	1.98	2.69	0.1427
Polyphenol oxidase (ΔA_{412}/mg protein/min)	0.0066	0.0179	0.0022
Titratable acidity (mg citric acid/g fr wt)	3.47	1.61	0.2731
pH	3.065	3.485	0.1833
Anthocyanin (ΔA_{540}/g fr wt)	0.531	0.321	0.0374
Phenols (mg/100 g fr wt)	1.380	1.075	0.0847
Ascorbic acid (mg/100 g fr wt)	9.71	4.64	0.7838
Protein (mg/100 g fr wt)	94.50	84.68	3.7617

lower in AB seeds, whereas the levels of starch, total sugars and reducing sugars were higher compared to healthy seeds. It was apparent that the decrease of amylase activity in AB seeds resulted in higher levels of starch. However, higher levels of both total and reducing sugars in AB seeds along with starch could be attributed to their incomplete utilization. Thus, the data showed that the seeds in AB arils had reduced viability. It is well established that plant hormones play a significant role in the processes that lead to mature fruits and viable seeds[18]. Hormones help in stimulating transport of nutrients through the phloem, modify the sink strength by stimulating its growth and making the developing fruit into a strong sink[19]. GA_3 influences source capacity by increasing photosynthetic potential of plants and also helps enhance sink strength by increasing assimilate transport, thus establishing its role in the source–sink system[19]. IAA, the most abundant endogenous auxin found in plants, is also known to enhance metabolite accumulation or increase sink strength[19]. Therefore, reduced levels of both GA_3 and IAA in the seed would make it a weaker sink and such seeds would be less able to attract assimilates towards themselves. As a result, these seeds would be unable to effectively compete with the neighbouring arils during fruit growth. Thus, localized inter-seed competition within a fruit and dominance due to production of growth regulators by the stronger sinks would lead to inhibition of growth in the weaker sink[20], leading to reduced seed viability.

Analysis of the juice from both H and AB-affected arils (Table 2) showed that the moisture content of the juice in the pulpy aril was higher in healthy (83.67%) compared to AB arils (81.13%). Biochemical analysis of the juice of H and AB arils showed that the levels of starch, TSS and pH were higher in AB compared to H arils, whereas TA, anthocyanins, total sugars, reducing sugars, total phenols, soluble protein and ascorbic acid were lower in AB compared to H arils. These data indicate that the process of degradation of starch to sugars occurring during fruit ripening was disturbed in AB-affected arils. The increase in pH from 3.065 in healthy juice to 3.485 in AB-affected arils reflects the reduction in the levels of organic acids in AB-affected arils, as a result of which TA is also reduced. The level of anthocyanins was found to reduce significantly in AB-affected arils, resulting in a reduction in the colour intensity. This is due to the fact that at the physiological pH of 3.0 in the plant vacuole, anthocyanins exist in a stable red flavylium ion form giving the arils their bright red colour, but as the pH increases to about 3.5, they undergo a reversible structural transformation to the anhydro base[21] forming colourless chromenols[22] and giving rise to arils with reduced colour intensity. The increase of PPO activity in AB-affected arils associated with a proportionate reduction in the levels of phenols indicated that browning in AB-affected arils was apparently due to the enzymatic oxidation of phenolic compounds by PPO to the highly reactive *o*-quinones, which form brown-coloured polymers[23] leading to fruit browning[24]. It has been reported that water loss in litchi fruit[25] led to an increase in the pH, causing an increase in the PPO activity and browning. PPO enzyme is known to travel through the thylakoid membrane[26] and act upon the substrate in the surrounding cytoplasm resulting in browning. In the present study also, pH was found to increase in AB-affected arils, whereas the moisture content showed a reduction compared to the H arils. As a result of such changes, an imbalance between oxidative and reductive processes would ensue leading to a loss of membrane integrity facilitating enzymatic oxidation of phenolic compounds to brown-coloured polymers and consequent browning reactions in the arils[27]. The reduction in the soluble protein content of AB-affected arils compared to the H arils is also indicative of the initiation of senescence reactions in AB arils[28].

From the foregoing, it is apparent that the onset of degradative processes in AB-affected arils is closely related to the changes in seed parameters, like moisture, TDH activity and seed viability. As discussed earlier, the reduction in seed moisture leads to a reduction in TDH activity and consequently the seed viability is affected.

In order to confirm the role of seed viability in AB formation, experiments were conducted on developing fruits at 50% and 80% maturity using preharvest application of PGRs. Results showed that GA_3 application to fruits increased TDH

Table 3. Total dehydrogenase activity and AB incidence (%) in fruits treated with plant growth regulators in the preharvest phase

Treatment	TDH activity	AB%
Control	2.9	18
GA_3-treated	3.16	5.51
PBZ-treated	1.39	32.25
CD (P = 0.01)	0.238	2.427

activity in the seeds and reduced the incidence of AB significantly, whereas PBZ application reduced TDH activity and markedly increased the AB incidence in 50% mature fruits (Table 3) compared to untreated control. Fruits applied with the growth hormones at 80% maturity stage did not show any differences in AB incidence compared to control. The increase of TDH activity in fruits treated with GA_3 and a marked reduction in PBZ-treated fruits compared to control fruits indicate that the applied growth hormones reach the seed, the target site, and influence seed viability in such a manner as to influence the incidence of AB, which is found to vary inversely with seed TDH activity. Previous studies with exogenously applied hormones consistently supported the conclusion that assimilates move towards regions of high hormone concentration, commonly referred to as hormone-directed transport by regulating sink strength[29]. Keeping the above fact in view, the data obtained in this study with regard to the incidence of AB in PGR-treated samples provide ample proof for a direct relationship between sink strength and seed viability, and confirm the causative role of seed viability in influencing AB incidence. Hence, it is evident that browning of arils occurring during AB development is initiated by a lack of seed viability.

Summing up, it is evident from the present study that during the early stage of fruit development, seeds of variable sink strengths evolve due to differences in the levels of growth hormones like GA_3 and IAA. The formation of strong and weak sinks among arils of a fruit leads to a reduction in the moisture content of weak sinks (seeds), resulting in either a reduction or complete loss of seed viability in them. The concurrent loss of moisture from the pulpy arils of such seeds results in the loss of membrane integrity followed by activation of PPO enzyme and the oxidation of phenols leading to AB. The present study has thus provided evidence for the direct role of seed viability in the development of AB in pomegranate. These results could therefore pave the way for successful management of the AB disorder in pomegranate by pre-harvest treatment of the fruits. It is possible that a number of fruit disorders bearing close resemblance to AB could also be caused by a mechanism similar to the one described here.

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