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Levels of phyto hormones during different stages of hip development in rose

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Abstract
Concentration of endogenous gibberellic acid (GA_3), abscisic acid (ABA) and indole-3 acetic acid (IAA) in achenes and pericarp of two varieties *viz*., 'Crifty Duty' and 'Five Star', were determined at weekly interval starting from 14th till 63rd days after pollination (DAP). GA_3 content in achenes initially increased till 35-42 DAP, while ABA was low during the period. In pericarp of both the varieties, ABA content was more than GA_3 while in achenes, ABA content was lower than that of GA_3. Proportion of GA_3 to ABA was found to be higher in achenes of 'Five Star' compared to that of 'Crifty Duty' except at 35 DAP, where in the proportion of GA_3 over ABA was more in 'Crifty Duty' compared to 'Five Star'. Maximum IAA content was recorded at 42 DAP and 35 DAP respectively in achenes of 'Crifty Duty' and 'Five Star' suggesting its role in embryo development.

Keywords: GA_3, ABA, IAA, rose, achenes

Introduction
Rose is the world's most popular flower due to its long history, symbolism, color, fragrance and sheer elegance of the form. There is a continuous demand for new rose varieties with varying color, size, fragrance as well as hardiness to biotic and abiotic stress. Hybridization to integrate desired characters from different sources is limited by inherent problems of seed germination. Seed germination in rose is reported to be constrained due to various reasons such as hard seed coat (Gudin *et al*., 1990, Bo *et al*. 1995) [4], hormones (Pipino *et al*., 2013,) [8] and genetic back ground (Anderson and Byrne, 2007) [1]. In *Rosa spp*., the mature embryo is protected by a seed coat called the testa, which forms the actual seed coat and is enclosed by the woody and hard pericarp (Zlesak, 2006) [12]. The structure of the rose seed is therefore botanically defined as an achene (Pipino *et al*., 2013) [8]. Fertilized flowers will develop in to fruits, which are called as hips (Reddy and Nagaraju, 2004) [10].

Hard seed coat is considered as major impediment in rose seed germination (Gudin *et al*., 1990) [4]. In rose, seed coat starts becoming hard as the days advances after pollination. Studies on rose embryo development have reported that embryo development completes at 15 days DAP (Pipino *et al*., 2013) [8]. Hence, the crucial decision required would be harvesting the seeds before the coat becomes hard and the embryo completes its development.

In addition to hardiness of seed coat, ABA is reported to be inhibiting rose seed germination (Yamba *et al*., 1992) [11]. ABA is considered the likely primary cause of physiological dormancy in roses (Yamba *et al*. 1992, Jin *et al*. 1995, Caser *et al*. 2012 and Pipino *et al*. 2013) [11, 6, 3, 8] while GA_3 is considered as one of the major hormone responsible for seed to germinate (Hosafci *et al.,* 2005, Pipino *et al.,* 2013 in rose) [5, 8]. Hence, it's important to decide the stage at which the absolute content of GA_3 is maximum while ABA is minimum. On the contrary it may also be possible that ratio between GA_3 over ABA may be the stage to be considered for harvest (Cadman *et al*., 2006) [2].

Ability of achene germination varies from variety to variety. In our rose breeding program, we have varieties with varying range of achene germination percentage. Variety 'Crifty Duty' has been observed to be having good germination percentage while the variety 'Five Star' is of poor germination percentage.

Based on the available reports of seed germination and our general observation of germination ability, we were trying to analyze the role of hormone in relation with the developmental stages of achene in variety 'Crifty Duty' that is with good germination percentage in

comparison with the variety 'Five Star' that is of poor germination percentage.

Materials and Methods

The present investigation was carried out at experimental form of ICAR-Indian Institute of Horticultural Research (IIHR), Hesaraghatta Lake Post, Bangaluru, situated at an altitude of 890 meter above mean sea level and latitude12^0 58´ north latitude, 78^0 45´ east longitudes respectively and temperature range between 25.33^0 C to 37.60^0 C. The experiment was laid out in Completely Randomized Design with eight treatment and three replications.

Plant material

Two rose varieties with maximum and minimum germination percentage in our rose breeding program were identified for the study. Variety 'Crifty Duty' has been observed to be having on an average of 30 per cent germination while the variety 'Five Star' is of poor germination with less than 1 per cent.

Table 1: Morphological character of 'Crifty Duty' and 'Five Star' rose varieties

Name of the cultivar	Flower number of petals	Flower diameter (cm)	Seed vessel	Hip shape of longitudinal section	Hip colour	Achenes per hips
Crifty Duty	Medium (20-30)	Large (8.1-10.0)	Large	Pitcher shaped	Yellow	8.00
Five Star	Many (>30)	Small (4.0-6.0)	Small	Funnel shaped	Brown	6.64

The best stage for pollination and seed set in rose was considered when two third of the flower petals were opened. To prevent self or unwanted pollination, the flowers were emasculated before the release of pollen in seed parents and flowers were bagged. The anthers from selected pollen parents (IIHRSG-1, Corvette, Bugathi, Konfetti, First Red, Gold Strike for Crifty Duty variety and Mirable, Sopha Gold, Odilla, Single Orange, Fancy Ruby Pink for Five Star variety) were collected in a petri dish lined with filter paper and placed uncovered at room temperature. The pollens released were collected 24 hrs later. Pollination was carried out in the morning (9-10 am) on emasculated flowers by brusing the pollen on the stigma possessing the sticky exudates using a fine paint brush. Pollinated flowers were labeled with the date of pollination, and hips were collected at 14, 21, 28, 35, 42, 49, 56 and 63 days after pollination (DAP). For each and every DAP considered for hormone estimation, sample collected consisted of around 15 hips.

Hips with labels collected at different DAP were wrapped in aluminum foil and transported in ice box to the lab for further analysis. The hips were cut into pieces and known quantity (5 g) was weighed for each sample. Pericarp of hips and achenes extracted from hips were crushed in mortar and pestle using liquid nitrogen under diffused light conditions till powder was consistent. Powdered samples were stored at -80^0C for further analysis.

Analysis of IAA, ABA and GA_3 by HPLC

Extraction medium of 80% methanol was prepared in distilled water and kept in refrigerator. The samples were mixed with 80 % ice cold methanol (15 ml) and transferred to 100 ml conical flask and kept overnight at 4ºC. On the following day, samples were filtered through what man filter paper No. 1 and filtrate was collected in a conical flask. The residue was re extracted in10 ml ice cold 80 % methanol and the supernatant was pooled and preserved at 4º C for further use. Aqueous methanol extract was placed in round bottom flask (100 ml) and the contents were dried at 45^0C under reduced pressure in Flash evaporator. The residue left was dissolved in 20 ml double distilled water and the pH of the aqueous extract was adjusted to 3 by adding few drops of 0.1 N HCl. The acidic extraction was transferred into a separatory funnel and equal volume of ice cold di-ethyl ether was added, shaken and the mixture was allowed to stand till the organic and aqueous phases separated out. The organic phase (di-ethyl ether) was separated out from the aqueous phase into a clean conical flask. This process was repeated three times and all the three aliquot of the organic phases were pooled together. A pinch of anhydrous sodium sulphate was added to the ether portion and contents were kept overnight at 4^0C to eliminate residual water. After filtration, ether was evaporated using Flash evaporator in V-shaped flask (100 ml). The residues were dissolved in 2.5 ml of HPLC grade methanol for HPLC analysis. The samples were placed in sterilized vials, sealed with paraffin film and stored in refrigerator for HPLC analysis. Before HPLC analysis, the sample was passed through 0.2 µm membrane filter discs in HPLC vials. HPLC vials used were cleaned with chromic acid and sterilized with HPLC grade methanol before filtration.

Results

GA_3 content differed significantly from 14 DAP to 63 DAP in both achenes and pericarp of 'Crifty Duty' (Table 2). In achenes and pericarp, GA_3 content ranged between 375 to 671 ng/g fw and 233 to 499 ng/g fw respectively. In achenes, maximum GA_3 content recorded at 35 DAP (671 ng/g fw) followed by 28 DAP (575 ng/g fw) and minimum GA_3 was recorded at 49 DAP (352 ng/g fw). In pericarp, GA_3 content was highest at 35 DAP (499 ng/g fw). Minimum GA_3 was recorded at 63 DAP (223 ng/g fw) in pericarp.

Significant differences were observed from 14 DAP to 63 DAP in both achenes and pericarp of 'Five Star' as well (Table.1). In achenes and pericarp, GA_3 content ranged between 334 to 881 ng/g fw and 353 to 568 ng/g fw respectively. Maximum GA_3 content was recorded at 42 DAP (881 ng/g fw) in achene followed by 35 DAP (798 ng/g fw) and 49 DAP (652 ng/g fw) and minimum was recorded at 14 DAP (334 ng/g fw). Maximum GA_3 was recorded at 35 DAP (568 ng/g fw). Minimum GA_3 was recorded at 63 DAP (353ng/g fw) followed in pericarp.

Abscisic acid (ABA) content showed significant differences from 14 days after pollination (DAP) to 63 DAP in both achenes and pericarp of 'Crifty Duty' (Table 3). In achenes and pericarp, ABA content ranged between 314 to 905 ng/g fw and 634 to 1009 ng/gfw, respectively. Maximum ABA content was recorded at 28 DAP (905 ng/g fw) in achenes followed by 14 DAP (748 ng/g fw). Maximum ABA content was recorded at 14 DAP (1009 ng/g fw) in pericarp. Minimum ABA content was recorded during 63 DAP (314 and 634 ng/g fw) in both achenes and pericarp, respectively. Significant variations were observed from 14 days after pollination (DAP) to 63 DAP in both achenes and pericarp of 'Five Star' (Table 2). In achenes and pericarp, ABA content ranged between 721 to 302 ng/g fw and 1368 to 885 ng/g fw, respectively. Maximum ABA content was recorded at 35 DAP (721ng/gfw) in achenes. While in pericarp, maximum ABA content was recorded at 21 DAP (1368 ng/g fw) followed by 28 DAP (1201 ng/g fw). Minimum ABA content

was recorded at 63 DAP (302 and 885 ng/g fw) followed by 49 (908 ng/g fw) DAP in both achene and pericarp, respectively. Significant variations in IAA content were observed from 14 DAP to 63 DAP in both achenes and pericarp of 'Crifty Duty' (Table 4). In achenes and pericarp, IAA content ranged between 231 to 356 ng/g fw and 176 to 262 ng/g fw, respectively. In achenes, maximum IAA content was recorded at 42 DAP (356 ng/g fw) and minimum was recorded at 14 DAP (231ng/g fw). While in pericarp, IAA content was maximum at 35 DAP (262ng/g fw) and minimum was recorded at 28 DAP (176 ng/g fw). Significant variations in IAA content were observed from 14 days after pollination (DAP) to 63 DAP in both achenes and pericarp of 'Five Star' (Table 3). In achenes and pericarp, IAA content ranged between 314 to 413 ng/g fw and 243 to 344 ng/g fw, respectively. Maximum IAA content was recorded at 35 DAP (413ng/g fw) followed by 28 DAP (387 ng/g fw) and minimum was recorded at 63 DAP (314ng/g fw) in achenes. While in pericarp, IAA content maximum was recorded at 56 DAP (344ng/g fw) and minimum was recorded at 42 DAP (243ng/g fw).

From the initial observation (14 DAP), an increasing trend in all the 3 hormone content was observed in achenes of both the varieties reaching a peak between 3rd to 6th week and then the hormonal concentration started decreasing. Gibberellic acid (GA_3) content in achenes was at peak on 35 days after pollination (DAP) in 'Crifty Duty' variety and on 42 DAP in var. 'Five Star'. This peak of GA_3 was associated with the drop of ABA (Fig.1).

In pericarp of both the varieties *viz.*, 'Crifty Duty' and 'Five Star', ABA content was much above $GA_3$and IBA was least compared to ABA and GA_3 at any time (DAP) of observation (Fig.1). In both the genotypes, GA_3content was on decreasing trend from 35 DAP onwards. In 'Five Star', GA_3 decrease started earlier (21DAP) compared to that in 'Crifty duty' (28 DAP). IAA content remained almost same compared to GA_3 and ABA levels in both the genotypes *viz.*, 'Crifty Duty' and 'Five Star' in both achenes and pericarp (Fig.1).

Relative content of GA_3 and ABA in 'Crifty Duty' and 'Five Star'

GA_3 content was high in achenes of 'Five Star' compared to 'Crifty Duty'. Even the ratio of GA_3 to ABA was found to be higher in achenes of 'Five Star' compared to that of 'Crifty Duty' except at 35 DAP, where in the proportion of GA_3 over ABA was more in 'Crifty Duty' compared to 'Five Star' (Fig. 2). Differences in GA_3 to ABA between the varieties was maximum at 42 DAP.

ABA content was higher in the pericarp of 'Five Star' as compared to 'Crifty Duty' pericarp. The ratio between ABA to GA_3 content in pericarp of 'Crifty Duty' and 'Five Star' was initially (14 DAP) high and peaked at 21 DAP, which started reducing and reaching lowest at 35 days. The ratio of ABA to GA_3 ratio increased further after 35 days (Fig. 3).

Table 2: Gibberellic acid (GA_3) concentration (ng/g fw) in developing hips of 'Crifty Duty' and 'Five Star'

Days after pollination	Varieties			
	Crifty Duty		Five Star	
	Achenes (ng/g fw)	Pericarp (ng/g fw)	Achenes (ng/g fw)	Pericarp (ng/g fw)
14	386 de	329 bc	334 g	383 de
21	447 cd	312 bc	489 f	402 d
28	575 b	465 a	632 c	472 c
35	671 a	499 a	798 b	568 a
42	562 b	342 b	881 a	534 b
49	352 e	278 c	652 c	457 c
56	375 e	301 bc	576 d	446 c
63	462 c	223 d	531 e	353 e
S.Em ±	23.57	17.45	12.60	10.23
C.D.@ 5%	70.68	52.33	37.79	30.68

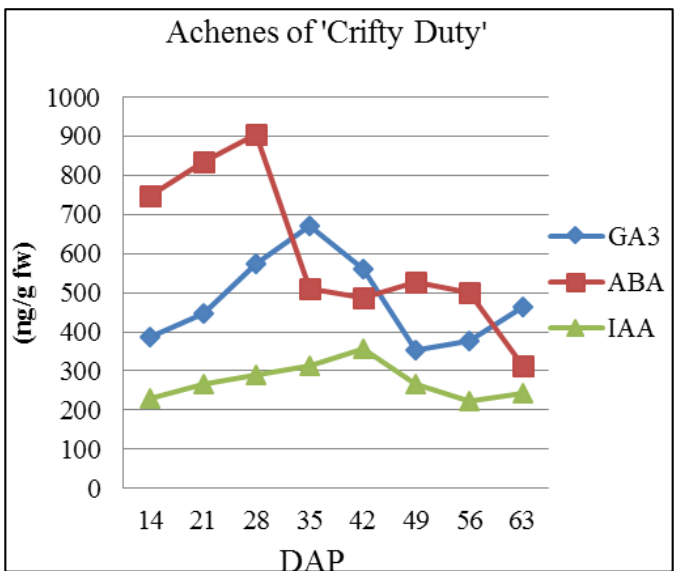
Table 3: Abscisic acid (ABA) concentration (ng/g fw) in developing hips of 'Crifty Duty' and 'Five Star'

Days after pollination	Varieties			
	Crifty Duty		Five Star	
	Achenes (ng/g fw)	Pericarp (ng/g fw)	Achenes (ng/g fw)	Pericarp (ng/g fw)
14	748 b	1009 a	564 b	1039 c
21	835 ab	963 ab	691 a	1368 a
28	905 a	813 c	703 a	1201 b
35	511 c	975 ab	721 a	1006 cd
42	486 c	907 b	386 c	989 cd
49	526 c	823 c	325 cd	908 cd
56	501 c	708 d	319 cd	954 cd
63	314 d	634 e	302 d	885 d
S.Em ±	31.80	24.20	24.11	46.81
C.D.@ 5%	95.63	72.55	72.30	140.34

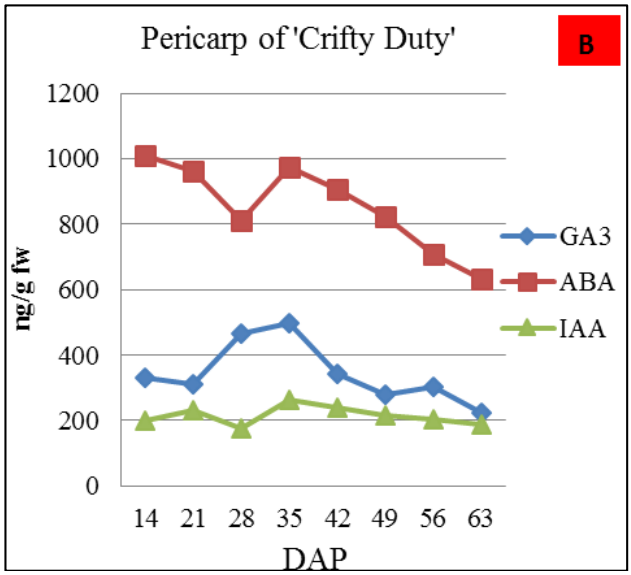
Table 4: Indole acetic acid (IAA) concentration (ng/g fw) in developing hips of 'Crifty Duty' and 'Five Star'

Days after pollination	Varieties			
	Crifty Duty		Five Star	
	Achenes (ng/g fw)	Pericarp (ng/g fw)	Achenes (ng/g fw)	Pericarp (ng/g fw)
14	231 d	198 cde	274 f	342 ab
21	265 bcd	233 abc	353 cd	295 cd
28	289 bc	176 e	387 ab	253 ef
35	314 ab	262 a	413 a	273 de

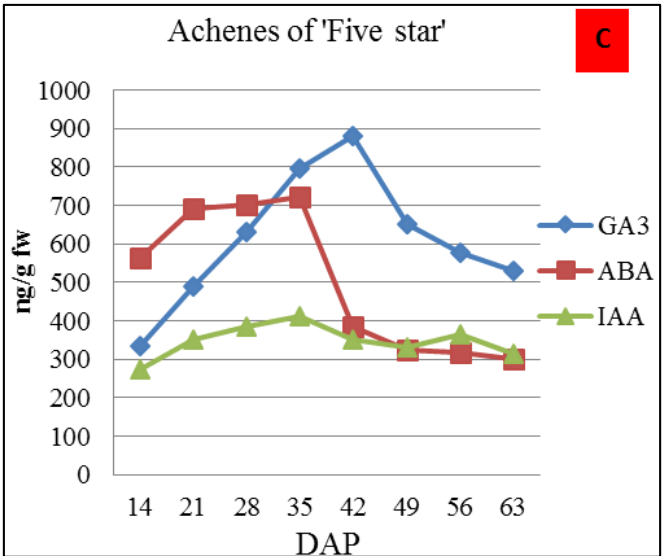
42	356 a	239 ab	351 cd	243 f
49	267 bcd	216 bcd	332 de	307 c
56	223 d	204 bcde	365 bc	344 a
63	243 cd	189 de	314 e	318.bc
S.Em ±	16.48	11.81	9.55	8.13
C.D.@ 5%	49.41	35.41	28.65	24.38



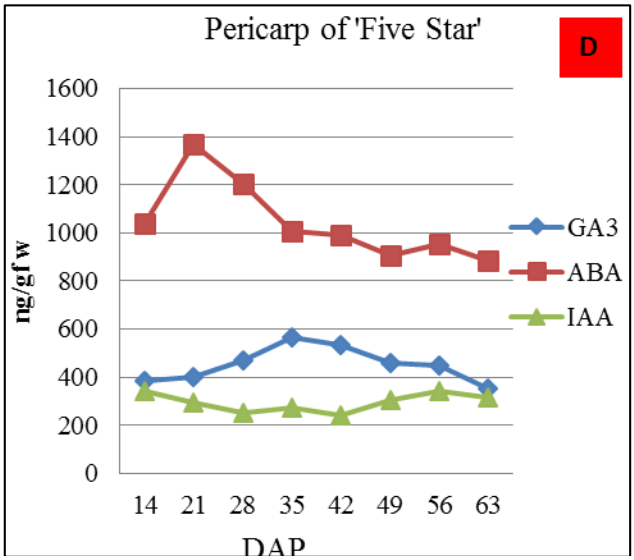
(A) Achenes of 'Crifty Duty'



(B) Pericarp of 'Crifty Duty'

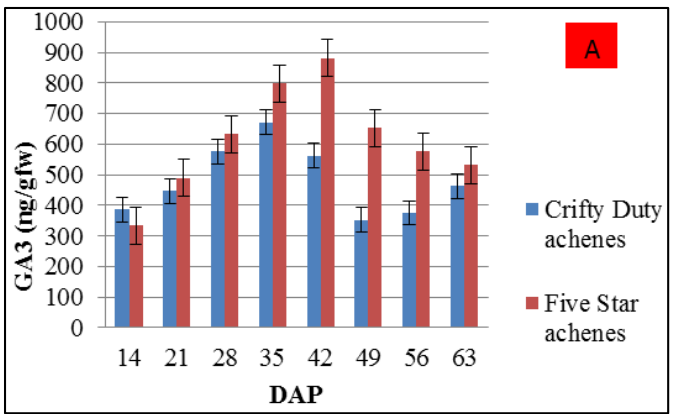


(C) Achenes of 'Five Star'

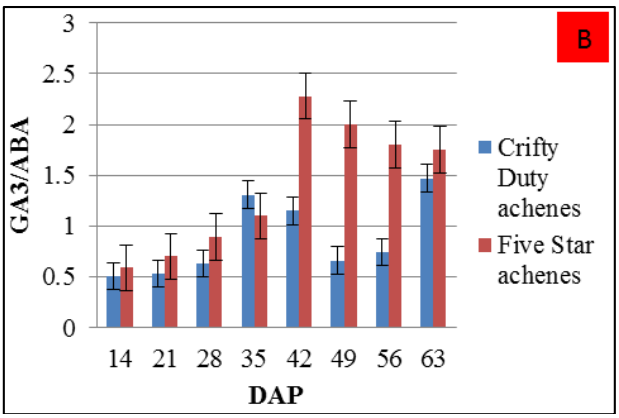


(D) Pericarp of 'Five Star'

Fig 1: Varying level of hormones in achenes and pericarp of 'Crifty Duty' and 'Five Star' at different days after pollination

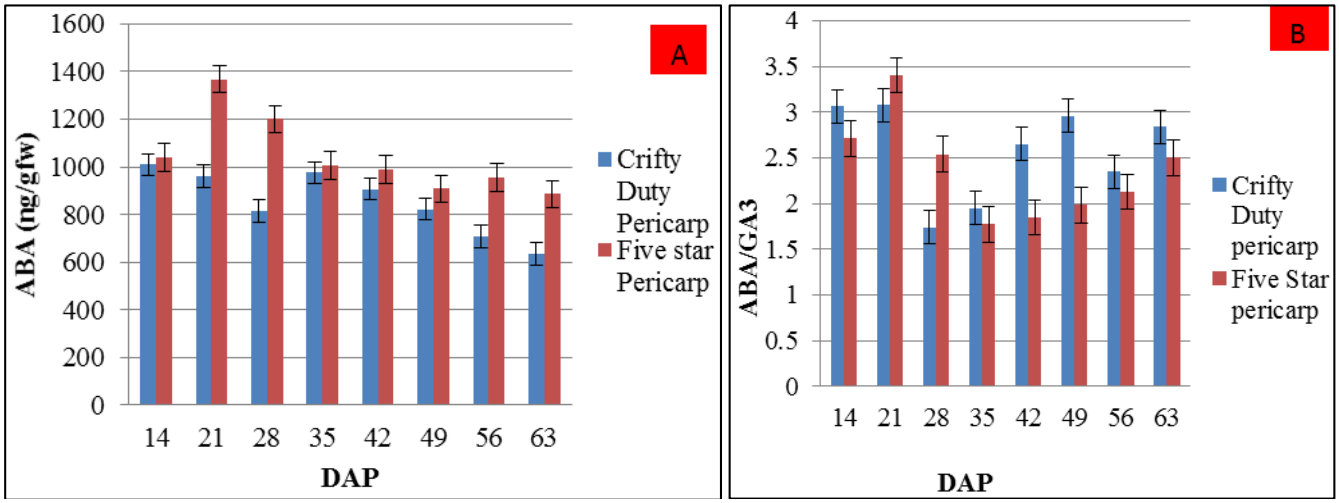


(A) GA_3 content in achenes of 'Crifty Duty' and 'Five Star'



(B) GA_3 /ABA inachenes of 'Crifty Duty' and 'Five Star'

Fig 2: Concentration of $GA_3$and Ratio of GA_3 over ABA (GA_3 / ABA) in achenes of 'CriftyDuty' and 'Five Star' during different days after pollination



(A) ABA content in pericarp of 'Crifty Duty' and 'Five Star' (B) ABA/GA_3 in 'Crifty Duty' and 'Five Star' in pericarp

Fig 3: Changes in the ABA/GA_3 in 'Crifty Duty' and 'Five Star' in pericarp

Discussion

If the absolute GA_3 content in achenes to be considered, at 35 days rose hips of 'Crifty Duty' can be harvested as that would also fulfill the need of embryo development as well as soft seed coat. On the contrary if we were to consider the proportion of GA_3 and ABA, then it may be ideal to harvest at 63 DAP, when ABA is at its minimum most and proportion of GA_3 over ABA is maximum. Earlier it was reported by Caser *et al*. (2012) [3] that the sizable drop in ABA content after full embryo development might be related to termination of the coat imposed seed physiological dormancy. ABA is considered the likely primary cause of physiological dormancy in roses (Yamba *et al*. 1992, Jin *et al*. 1995, Caser *et al*. 2012 and Pipino *et al*. 2013) [11, 6, 3, 8]. However, hard seed coat becomes the issue of concern. 'Crifty Duty' achenes were advisable for harvest at 35 DAP than 63 DAP. But in 'Five Star', achenes can be harvested at 42 DAP because GA_3 content was high at this stage compared to other DAP.

If hormones were considered to be the absolute reason for germination, then we could have got more germination in 'Five Star' compared to that of 'Crifty Duty' as the results indicated higher amount of GA_3 and lesser amount of ABA in 'Five Star' compared to 'Crifty Duty'. Trend of GA_3 in achenes and pericarp remained same over the period across varieties, in both 'Crifty Duty' as well as 'Five Star'. On the contrary ABA trend differed between achenes and pericarp. ABA content was more in achenes of 'Crifty Duty' compared to 'Five Star', while in pericarp, ABA content was more in 'Five Star' than 'Crifty Duty'. Low germination in 'Five Star' compared to 'Crifty Duty' cannot be attributed to ABA level in achenes and though pericarp Five Star has high ABA, in the seed germination procedure, we always remove pericarp, hence even though it's high, it cannot be attributed as the reason for poor germination in Five Star. Even when we looked at the results of proportion of GA over ABA in achenes, its high in 'Five Star' compared to 'Crifty Duty'. So, difference in response of genotypes for germination cannot be limited to hormone content. ABA content decreased in both achenes and pericarp at 35 DAP in 'Crifty Duty' and 'Five Star'. ABA content was recorded maximum in pericarp of 'Five Star'. Similar result was reported in hips of rose varieties 'Melglory' and 'Cassandra' (Caser *et al.*, 2012) [3]. ABA concentrations in the embryos were significantly reduced at 30 DAP when seeds were fully formed (Caser *et al.*, 2012) [3].

Recently, Pipino *et al*. (2013) [8] studied ABA concentrations in developing embryos of two floribunda roses, 'Melglory' and 'Cassandra'. The high ABA levels were found during the early developmental torpedo stage (9 DAP). At 30 DAP, the same hips contained fully formed seeds and embryo with significantly reduced ABA concentrations. From these results, it is suggested that initial high ABA concentration was involved in the inhibition of precocious germination and same is supported in the finders of Pipino and co-authors (2013) [8]. In the present study, a similar trend of high ABA content in the early developmental stage followed by decreasing trend starting from 28-35 DAP was observed in both achenes and pericarp of 'Crifty Duty' and 'Five Star'. The difference in ABA concentration between genotypes could be related to the genetic makeup of seed parent of the ABA levels during early development (Raz *et al*., 2001) [9].

The ratio GA_3 to ABA in 'Crifty Duty' and 'Five Star' achenes was >1, enhancing the seed maturation and seed germination. In accordance with the most studied relationship between ABA and GA_3 during seed maturation, crosstalk between ABA and IAA during seed development and in particular during germination, has been reported in *A. thaliana* (Liu *et al*., 2007) [7] (Fig. 2). The ratio ABA to GA_3 was increased with the pericarp development in both 'Crifty Duty' and 'Five Star'. ABA to GA_3 ratio, and not the absolute hormone contents, which controls dormancy and germination by antagonistic effects of both the hormones. ABA inhibits the GA_3 induced synthesis of hydrolytic enzymes essential for the activation of storage compounds in germinating seeds, has been reported in *A. thaliana* (Cadman *et al*., 2006) [2] (Fig.3).

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