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Research Article

ABIOTIC ELICITOR MEDIATED AUGMENTATION OF ANNATTO PIGMENT PRODUCTION IN STANDING CROP OF *BIXA ORELLANA* L.

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ABSTRACT
Abiotic elicitors methyl jasmonate (MJ), salicylic acid (SA) and paclobutrazol (PB) have shown significant improvement in total annatto pigment content of seeds of *Bixa orellana* that subjected to floral spray. Up to 2 and 1.5 to 2 folds of improvement in total annatto pigment content and % bixin levels was achieved through abiotic elicitors MJ, SA and PB spray respectively. Various growth regulators *viz*., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), gibberellic acid (GA_3), and 2,3,5-triiodobenzoic acid (TIBA) spray leads to moderate improvement in annatto pigment. Similarly chitin or chitosan spray resulted in 1.5 to 2 folds improvement in total annatto pigment and % bixin levels. All the three elicitors MJ, SA and PB were tested for three flowering seasons consecutively (2007, 2008, and 2009) and found consistence in response for annatto pigment augmentation. Hence, elicitor mediated augmentation of annatto pigment would have a wider scope for commercial purposes.

Key Words: *Bixa Orellana, Elicitor, Methyl Jasmonate, Paclobutrazole, Salicylic Acid*

INTRODUCTION
Annatto a reddish orange pigment of plant origin, is a pulpy, resinous coating derived from the arils of seed of *Bixa orellana* L. (Bixaceae) which is an evergreen shrub of 2 - 5 m height, that is native to tropical America (Wealth of India, 1990) and now grown in most of the tropical countries Bolivia, The Dominican Republic, Ecuador, Guyana, India, Jamaica, Mexico, and Peru etc. The crop is also produced on smaller degree in Africa, e.g., Kenya, Nigeria, Tanzania and in the pacific e.g., Phillippines and Hawaii. In India its cultivation is familiar in the forest regions of Andhra Pradesh particularly in coastal districts Visakhapatnam and Srikakulam along with Thiruvananthpuram (Kerala), Malabar and Coromandal coasts and in some places of Karnataka, Madhya Pradesh, Maharashtra, Orissa and West Bengal. In view of annatto dye's wide range of applications in food industry and also in cosmetics and dyeing of leather (Wealth of India, 1990), annatto seeds have a vivid prospect of marketing in India and abroad (Satyanarayana *et al.,* 2003). Though India is not basically export-oriented producer, of Bixa, during the past two decades to some extent ordered farming of annatto crop became popular mainly in southern parts of India (Aparnathi *et al.,* 1990). In contrary to this globally the bulk of the annatto crop is achieved from wild trees or casual cultivation in Peru and Kenya respectively. The sunny climate with seasonal rains are the conducive factors suitable for Agroforestry of Bixa in Southern states of India. In contrast to the 3-4% of total pigment content of seeds from Peru, in India it varies from 0.73 - 1.5% with 82% of this as bixin- a bright reddish apocarotenoid, which is quite low to the international bench mark of ~ 2.5% of bixin in order to get good price at global market (ITC, 1994). In view of this, the improvement of annatto pigment content of seeds of *B. orellana* standing crop in India is warranted. In this regard, elicitor mediated technology would be having greater advantage as both biotic or abiotic elicitors are known for their role augmentation of secondary metabolites in *in vitro* cultures of plants (Tamari *et al.,* 1995; Bhuiyan and Adachi 2003; Sudha and Ravishankar 2003). In view of this an effort has been made to augment annatto pigment production in standing crop of *B. orellana* L. by using various abiotic elicitors such as methyl jasmonate, salicylic acid and paclobutazol along with some growth regulators that partially covered under Indian Patent (Parmialan *et al.,* 2005). We are herewith reporting the improvement in total annatto pigment content production under elicitor treatments in standing crop of *B. orellana*.

MATERIALS AND METHODS

Field establishment, elicitor preparation and spray

Three years old plants of *Bixa orellana* L. that bear pink flowers and red obovate fruits (landrace) were used for a full-fledged small-scale field study for improving the annatto pigment on standing crop through elicitor mediated technology. Different plants were selected for spraying various elicitors in our study. All these plants were under farming in an area of 100 X 80 ft at the back yard of Plant Cell Biotechnology Department, of this institute, with a spacing of 4 x 4 ft between each plant. Plant growth regulators viz., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), N-(2-Chloro-4-pyridyl)-N'phenylurea (CPPU), gibberellic acid (GA_3), α-naphthalene acetic acid (NAA), 2,4,5-Triiodobenzoic acid (TIBA) and triacontanol (TRIA), along with abiotic elicitors such as methyl jasmonate (MJ), salicylic acid (SA), paclobutrazol (PB) , chitosan, and ibuprofen were used to identify in a broad way for its utility towards elicitation. All these growth regulators, their inhibitors, MJ, SA and PB were procured from Sigma, St. Louis, MO, USA. Abiotic elicitors MJ, SA and PB were selected further for ontogeny analysis towards its annatto pigment content. Respective elicitor stocks were prepared at 10 mM concentration and they were diluted to working concentrations (1, 2,5, 5 or 10 µM) using distilled water and were sprayed to the flowers during anthesis (between 9-11 Hrs) and the flowers were tagged. Similarly MJ, SA and PB at 0.5 to 5µM concentrations were sprayed to the flower on the day of anthesis. The annatto pigment content of the seeds from tagged fruits was analyzed at 10 days interval till maturity for studying its effect on optimal enhancement during fruit development. Ontogeny experiment was carried out for three consecutive flowering seasons (yearly flowering) in order to find out the consistency of response for respective elicitor spray. The fruits of respective treatments were harvested at maturity (after 60d).

Their total color content and % bixin content were analyzed by using spectrophotometric method as reported by Mc Keown and Mark, (1962).

$$\text{Total pigment (g/100g)} = \frac{\{A_{500} + A_{404} - 0.256(A_{500})\} * V * 100}{282.6 * 1000 * W}$$

Where, A_{500} is absorption at 500nm
A_{404} is absorption at 404nm
V is final volume
W is weight of the sample taken

Similarly the bixin content of the annatto colour in terms of g per 100g was calculated.

$$\text{BIXIN} = (A_{500\,max} / 282.6) \times (V2/V1) \times (V3/1000) \times (100/wt).$$

For example V2= 5.0, V1= 0.1 and V3 = 50 are the aliquot used, volumes of the sample solution, and the final solution. The absorptivity of bixin is 282.6 at 501 mµ. wt is the weight of the sample in grams.

Statistical analysis

The entire experiment was conducted in a randomized block design with five replicates. For each treatment fifty flowers were sprayed. Five fruits of respective tagged flowers were taken for analysis of total annatto pigment content at every 10 days for each and every treatment. Data obtained were subjected to statistical analyses for the significance of the study using one-way analysis of variance and the means were separated using Duncan's multiple range test. The best concentrations of respective growth regulators for maximum augmentation of annatto pigment were provided in table1 and for MJ, SA and PB the annatto pigment' augmentation at all three treatment levels of study were provided in Table 2.

RESULTS AND DISCUSSION

Among the hormones, inhibitors and abiotic elicitors tested, best three elicitors were selected and used further to study the enhancement in pigment content throughout the ontogeny of fruit. Growth regulators such as IAA, IBA, NAA, CPPU and TRIA, showed mild to moderate response wherein, 1.0 to 1.6 folds

improvement in annatto pigment production was evident in seeds that harvested 60 days post-anthesis (Table 1). Similarly chitin and chitosan induced 1.5 to 1.71 folds improvement in pigment production. Paclobutrazol (PB) spray at 1.0 µM concentration resulted in a maximum of up to 2 folds (Table 1) increase of annatto pigment content. A similar response was found with both 1.0 µM MJ and PB floral spray respectively for annatto pigment augmentation. A perusal of table 2 reveals about the consistency in annatto pigment augmentation under respective elicitors spray for 3 consecutive years. When all the three elicitors were tried even at low levels i.e 0.5 µM, the % annatto pigment content was almost same that of respective controls i.e. distilled water spray (data not given). Unlike MJ and SA, PB floral spray at all the three concentrations i.e. 1.0, 2.5 and 5 µM improved the % total pigment content in seeds to more that 4%. Even the % bixin of this total annatto pigment content was high (>3.2%) compared to MJ and SA.

Table 1: Influence of different abiotic elicitors on annatto color content in seeds of *B. orellana*

S. No	Name of elicitor	No. of folds of pigment enhancement*	Optimal concentration of the elicitor gave maximum folds of pigment (µM)
1	Chitin	$1.5^{d,e}$	1.0
2	Methyl Jasmonate	4.36^{a}	10.0
3	Paclobutrazol	$3.39^{a,b}$	10.0
4	GA_3	2.03^{b}	1.0
5	Chiotosan	1.71^{c}	2.5
6	Indole-3-butyric acid	1.66^{c}	1.0
7	CPPU	$1.6^{c,d}$	2.5
8	Ibuprofen	$1.6^{c,d}$	2.5
9	Salicylic acid	$1.3^{f,g}$	2.5
10	Naphthalene acetic acid	$1.36^{e,f}$	2.5
11	Indole-3-acetic acid	$1.35^{e,f}$	1.0
12	Triacontanol	1.2^{g}	2.5
13	TIBA	0.9^{h}	2.5

**Same alphabets between rows within the column are not significant ($p<0.05$); values are an average of five samples (± S.E.)*

There was marginal enhancement in % annatto pigment in MJ , SA and PB treatments in second flowering season (the year 2008) compared to first flowering season (the year 2007). But in the third year again there was slight reduction in % annatto pigment. But statistically the decrease or increase is insignificant with an overall improvement of up to 2 folds in pigment production. A similar trend was noticed in % bixin levels during the three flowering seasons. There was a gradual increase in annatto pigment production and % bixin levels during the ontogeny of fruit with maximum pigment content during 40-60days (Table 3 and 4). The trend was same in both control and elicitor treatments. The pigment content rather stabilized or decreased by 70 days (data not given) because of the seeds get exposed to outside environment upon fruit dehiscence. It may be noted that the % annatto pigment content in controls (no elicitor spray) was 2.164 % in seeds harvested at 60 days post anthesis. But under 1-2.5 µM PB spray the same 2% annatto pigment was noticed in seeds by 40 days post anthesis and by 60 days the % annatto pigment reached to 4.4%. Both MJ and SA spray also showed a similar response for both annatto pigment content (Table 3) and also for % bixin content (Table 4).

Table 2: Annatto pigment content during three consecutive seasons (2007, 2008, and 2009) in standing crop of *B. orellana*

Elicitor	Treatment levels (µM)	2007 study		2008 study		2009 study	
		% total annatto pigment*	% Bixin*	% total annatto pigment*	% Bixin*	% total annatto pigment*	% Bixin*
MJ	Control	2.67 ±0.19^{c}	1.80 ±0.15^{c}	2.187±0.08^{b}	2.02 ±0.01^{d}	1.90 ±0.008^{b}	1.67 ±0.05^{c}
	1	4.281 ±0.28^{a}	3.32 ±0.12^{a}	4.70 ±0.07^{b}	3.81 ±0.01^{a}	4.18 ±0.07^{a}	3.60 ±0.09^{a}
	2.5	3.66±0.17^{b}	2.85 ±0.08^{b}	3.80 0.05^{b}	2.77 ±0.02^{c}	3.89 ±0.05^{a}	3.12 ±0.07^{b}
	5	3.48±0.19^{b}	2.65 ±0.07^{b}	3.95 ±0.07^{b}	3.15 ±0.01^{b}	4.15 ±0.05^{a}	3.18 ±0.05^{b}
SA	Control	2.12 ±0.08^{cd}	1.98 ±0.01^{c}	2.24 ±0.01^{c}	1.98 ±0.01^{c}	2.0 ±0.02^{c}	1.78 ±0.01^{c}
	1	4.05 ±0.20^{a}	3.21 ±0.12^{a}	4.20 ±0.03^{b}	3.66 ±0.01^{a}	3.91 ±0.04^{a}	3.24 ±0.12^{a}
	2.5	3.31 ±0.02^{bc}	2.84 ±0.18^{b}	3.45 ±0.02^{c}	2.92 ±0.07^{b}	3.79 ±0.02^{a}	3.15 ±0.09^{a}
	5	3.086 ±0.32a	2.674 ±0.02^{b}	3.167±0.08^{b}	2.79±0.010^{b}	3.08 ±0.014^{b}	2.58±0.075^{b}
PB	Control	2.16 ±0.09^{c}	1.70 ±0.04^{b}	2.13 ±0.06^{c}	1.86 ±0.01^{c}	1.98 ±0.01^{b}	1.65 ±0.01^{c}
	1	4.41 ±0.14^{a}	3.65 ±0.18^{a}	4.56 ±0.04^{b}	3.77 ±0.01^{a}	3.91 ±0.03^{a}	3.19 ±0.06^{b}
	2.5	4.39±0.19^{ab}	3.54 ±0.16^{a}	4.45 ±0.25^{a}	3.59 ±0.01^{ab}	4.10 ±0.02^{a}	3.61 ±0.14^{a}
	5	4.25 ±0.28^{b}	3.42 ±0.12^{a}	4.13 ±0.03^{b}	3.31 ±0.01^{b}	4.21 ±0.02^{a}	3.55 ±0.17^{a}

MJ= Methyl jasmonate; Sa= Salicylic acid; PB= Paclobutrazol
**Same alphabets between treatments levels of major rows (elicitors) in a column are not significant ($p<0.05$); values are an average of five samples (± S.E.)*

Statistical analysis revealed that there is no significance between replicates and also between three consecutive years for any single treatment. There was significance between treatments of varied concentrations and type of elicitors. Since different elicitors vary differently in their response, means of treatment were separated physically based on the type of elicitor used and within which they are separated using DMRT and the same has been reported in Table 2.

The elicitor type activity of PB – a triazole compound was supported a similar studies (Zhang and Franco 2005; Gopi *et al.*, 2007). Though, paclobutrazol was used as gibberellic acid biosynthesis inhibitor (Davis and Curry 1991); elicitation with gibberellic acid also enhanced the total annatto pigment content by 2.03 folds (table 1). The reason may be due to the fact that, presence of paclobutrazol inhibits GA biosynthesis. The mode of action was known to be the inhibition of *ent*-kaurene oxidase – the enzyme that catalyses the sequential oxidations from *ent*-kaurene to *ent*-kaurenoic acid – in the early sequence of GA biosynthesis (Graebe, 1987; Rademacher *et al.*, 1987). Since *ent*-kaurene is formed from geranylgeranyl pyrophosphate (MacMillan 1997); when paclobutrazol is sprayed, due to inhibition of the

enzyme *ent*-kaurene oxidase, endogenous levels of *ent*-kaurene gets increased and thereby through end-product inhibition endogenous biosynthesis of *ent*-kaurene will be inhibited, so there may be increase in the endogenous pools of GGPP. This increase in endogenous levels of GGPP might be diverted to carotenoid biosynthesis so as to maintain equilibrium of different components at cellular level. This might form the basic reason for the action of paclobutrazol on the enhancement of carotenoids with special reference to annatto pigment. Simultaneously, since we also sprayed GA_3, it was expected for reduction in pigment content rather, it had enhanced the annatto pigment content to 2.03 folds (Table 1).

Table 3: Total colour content of annatto pigment during the ontogeny of fruit in *B. orellana* under abiotic elicitor spray during the flowering season 2007

Elicitor	Treatment levels (µM)	% of total annatto pigment*		
		20d	40d	60d
Paclobutrazol	Control	0.85±0.13 [a]	0.94±0.08 [c]	2.16±0.09 [b]
	1	0.84±0.14 [a]	2.12±0.07 [a]	4.41±0.14 [a]
	2.5	0.90±0.10 [a]	2.26±0.08 [a]	4.39±0.19 [a]
	5	0.42±0.09 [b]	1.60±0.07 [b]	4.25±0.28 [a]
Methyl Jasmonate	Control	0.72±0.02 [b]	1.34±0.06 [b]	3.67±0.19 [b]
	1	0.23±0.01 [c]	3.57±0.26 [a]	4.28±0.28 [a]
	2.5	1.02±0.08 [a]	1.28±0.18 [b,c]	3.66±0.17 [b]
	5	0.23±0.09[c]	1.16±0.10 [c]	3.48±0.19 [c]
Salicylic Acid	Control	0.73±0.02 [b]	1.10±0.06 [c]	2.0±0.015 [c]
	1	0.21±0.08 [c]	2.98±0.32 [a]	3.91± 0.04[a]
	2.5	0.98±0.06 [a]	1.37±0.25 [b]	3.79± 0.03[a]
	5	0.23±0.08 [c]	1.38±0.21 [b]	3.08±0.014[b]

**Same alphabets between treatments levels of major rows (elicitors) in a column are not significant (p<0.05); values are an average of five samples (± S.E.)*

Table 4: Percentage bixin content of harvested seeds for *B. orellana*

Elicitors	Treatment levels (µM)	% of total annatto pigment*		
		20d	40d	60d
Paclobutrazol	Control	0.68± 0.10[a]	0.80± 0.14[c]	1.70± 0.21[b]
	1	0.67± 0.09[a]	1.85± 0.12[a]	3.65± 0.45[a]
	2.5	0.72± 0.07[a]	1.89± 0.18[a]	3.54± 0.28[a]
	5	0.32± 0.05[b]	1.25± 0.24[b]	3.42± 0.34[a]
Methyl Jasmonate	Control	0.56± 0.08[b]	0.99± 0.28[b]	2.80± 0.18[b]
	1	0.16± 0.01[c]	2.60± 0.35[a]	3.32± 0.22[a]
	2.5	0.82± 0.18[a]	0.99± 0.28[b]	2.85± 0.18[b]
	5	0.17± 0.04[c]	0.90± 0.35[b]	2.65± 0.35[c]
Salicylic Acid	Control	0.56± 0.06[b]	0.87± 0.22[c]	1.78± 0.18[c]
	1	0.16± 0.03[d]	2.45± 0.18[a]	3.24± 0.24[a]
	2.5	0.72± 0.05[a]	1.25± 0.09[b]	3.15±0.28[a]
	5	0.28± 0.03[c]	1.05± 0.05[b]	2.58±0.20[b]

**Same alphabets between treatments levels of major rows (elicitors) in a column are not significant (p<0.05); values are an average of five samples (± S.E.)*

This could be due to increase the endogenous pools of GA_3 under GA_3 spray, and thereby GA biosynthesis might be blocked through end-product inhibition and hence resulting in increased levels of *ent*-kaurene – the precursor of GA – and thereby blocking the biosynthesis (Rohmer *et al.,* 1993). Due to the similarity of the biosynthetic pathway for GA and carotenoids involving DOXP pathway for formation of GGPP – the precursor for both GA and carotenoids, blocking in GA biosynthesis diverts the precursor to carotenoid biosynthesis – the very next alternate pathway. This forms the reason and the mechanism by which the enhancement of annatto pigment occurs while using the two compounds of paradoxical nature that ends in same results – the enhancement of compounds in carotenoid biosynthesis and in this case, annatto pigment. However, molecular evidences are yet to confirm this mechanism.

Both MJ and SA are known for their elicitor activity particularly by augmenting various secondary metabolites produced by plant cell cultures. In general plants produce jasmonic acid and methyl jasmonate in response to biotic and abiotic stresses, which accumulates in the damaged parts of the plant. The same MJ is also demonstrated as best abiotic elicitor. Treatment with jasmonates can elicit the accumulation of several classes of alkaloids (Zabetakis *et al.,* 1999), phenolics (Lee *et al.,* 1997) activation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco (Sharan et al., 1998) and betacyanin synthesis in *Portuluc*a (Bhuiyan and Adachi 2003) and capsacin in *Capsicum* (Sudha and Ravishankar 2003). Apart from this Tamari et al (1995) showed that methyl jasmonate induces pigmentation and flavonoid gene expression in Petunia. Moreover, a mechanism by which MJ induced-gene expression involved in plant secondary metabolites biosynthesis at molecular level was demonstrated (van der Fits and Menelink 2000; Suzuki *et al.,* 2005). But such reports are not scanty with reference to augmentation of caroteonoids. Augmentation of annatto an apocarotenoid of *B. orellana* by MJ in our study was supported by a recent report wherein MJ enhanced carotenoid content in yellow pigmented cut rose flowers (Glick *et al.,* 2007). Hence, our study indicated that floral application of MJ could increase the annatto pigment content in *B. orellana* as discussed in this work. Salicylic acid is a vital component of the plant resistance to pathogens and play a part in the plant response to adverse environmental conditions. Similarly salicylic acid is also known for its potential to improve carotenoids (Czerpak *et al.,* 2003; Moharekar *et al.,* 2003; Çag *et al.,* 2009). Recent studies have demonstrated about the importance of SA in isoprenoids (precursors of carotenoids pathway) production and accumulation (Çag *et al.,* 2009).Though different plant growth regulators have showed moderate response for augmenting annatto pigment, this response was further supported by similar studies with IAA and NAA that improved the cartoenoids profiles in plants (Czerpak *et al.,* 2003). Ibuprofen is a lipoxygenase inhibitor (Menke *et al.,* 1999) and also shown earlier as a potent inhibitor of elicitor-induced phytoalexin production in rice suspension cultures (Nojiri *et al.,* 1996). In our study its spray at 2.5 µM leads to moderate increase in annatto pigment content, though there is no evidence of its role in carotenoids augmentation.

TRIA is known on for its growth regulation properties in plants and also for improving secondary metabolites production (Giridhar *et al.,* 2005). The response of 2.5 µM of Triacontanol towards annatto pigment augmentation is further supported by similar reports in tomato wherein, 1 µM TRIA with potassium augmented both lycopene and betacarotenes by 8-9% (Khan *et al.,* 2009). Chitin and chitosan are known for their elicitor activity (Dutta *et al.,* 2004) and both are able to improve annatto 1.5 to 2.0 folds of pigment production in *B. orellana* seeds in our study.

Another important observation in our study was fruit dehiscence. In general 55-60days post-anthesis the fruits become dry and break, so that seeds can be harvested. In controls (with out any elicitor spray) the same phenomenon was noticed. But under MJ, SA and PB floral sprays, in the respective fruits the dehiscence was delayed by 20-25 days, which is a good sign especially it could be useful to escape unfavourable environmental conditions such as unseasonal rains that could create favourable conditions for seed mycoflora which hampers seed loss and quality. In our study, annatto pigment content was estimated once in ten days to monitor the optimal duration for enhancement. The results hence obtained showed that % annatto pigment in control plant seeds of 60 days post anthesis could be achieved by 40-50

days of post anthesis in seeds of respective elicitor (MJ, SA or PB) treated plants, an the same would reach to ~ 4% by 60 days post anthesis. This methodology can be used for the standing crop for enhancement of annatto pigment content without replacing the low yielding trees by high yielding trees that costs more and also takes three years to start flowering and fruiting.

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