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AZADIRACHTA INDICA A. JUSS. (NEEM): INFLUENCE OF GA_3 AND KINETIN ON MORPHO-PHYSIOLOGICAL CHARACTERISTICS

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SUMMARY

The *in vivo* morpho-physiological responses to GA_3 and kinetin application in Neem (*A. indica*) are studied during annual reproductive flushes from bud development onwards to seed set. The buds and flowers per inflorescence were higher in kinetin treated branches as compared to control. Protein, total sugars and reducing sugars measured higher following kinetin treatment as compared to GA_3. GA_3 treatments produced poor flowering response, longer juvenility and low contents of the above biochemical analyses. The enzyme assay of invertase, protease, acid- and alkaline phosphatases matched the biochemical responses to the growth regulator treatments. In control, a shorter regeneration phase leads to lower productivity of floral inflorescence and fruit set. Therefore, it is suggested that the application of kinetin may regulate the reproductive phase resulting in improved morpho-physiological characteristics.

***Key words*: *Azadirachta indica*, GA_3, kinetin, neem, reproduction**

INTRODUCTION

Neem, a prodigious multipurpose tree of the tropics, has immense potential to benefit mankind and to protect the environment (Kraus 2002, Kaaya *et al.* 2003). Often called 'Gift of the Gods' or 'Nature's Pharmacy', the tree is exploited as a commercial medicine and antibiotic. Extracts from its extremely bitter seeds and fruit may infact be the source of a new generation of chemicals for use in Integrated Pest Management practices (Walia *et al.* 2002, Koul and Wahab 2007).

In recent years, foliar application of plant growth regulators on cash crops has enhanced plant growth, development and yield (Vila *et al.* 2004). Kinetin and GA_3 have now been recognised as plant growth regulators which can mediate reproductive differentiation in various plants (Dewittee *et al.* 1999, Vila *et al.* 2004). These reports are based on their ubiquity, abundance in growing tissues and their effects on growth and development (Kaminek *et al.* 1997, Durdan *et al.* 2000). In addition, to various responses produced at molecular level, the growth regulators influence protein, nucleic acid and mitotic activity (Dhir *et al.* 1986, Letham, 1994, Metzger, 1995, Koul and Wahab 2007) and also play a role in cell division, growth and induction of invertase and phosphatase syntheses (Pandey *et al.* 2000, Vila *et al.* 2004).

However, most of the information is restricted to the vegetative phase of a plant and there is little information with respect to reproductive phase, especially on fruit. The present study investigates the effect of kinetin and GA_3 during reproductive growth of Neem with the aim to determine the effect of exogenously applied kinetin and GA_3 on floral anthesis and fruit development,

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associated with primary metabolic pathway and explore a possible role of growth regulators in increasing the reproductive potential.

MATERIALS AND METHODS

The experiment was conducted in the pharmaceutical garden nursery in Panjab University, Chandigarh (Lat. 30.5 N Long. 77.0 E). Young reproductively mature trees of 5-6 years age were selected. Just prior to the onset of the regeneration phase in March, apices of the test branches were sprayed *in vivo* with standardised kinetin and GA_3 solutions with a few drops of Tween 20.

Preliminary experimentation: In accordance to previous test analyses and studies (Sokal and Rohlf 1973, Devkumar and Sukhdev 1993), the growth regulators were applied. The number of reproductive structures per panicle, their size, weight and quality were recorded and the best treatments were correlated and standardised (Sabherwal 2000).

Main experimentation: In the present study, GA_3 and kinetin in the concentrations 0.5 mM and 0.2 mM were applied to the test branches. Observations were recorded separately at various stages of reproductive development *viz*, bud sprout/pre-anthesis (S_1), flower bloom/anthesis (S_2), fruit set/post-anthesis (S_3) and fruit senescence/seed maturation (S_4). Extraction and estimation of total sugars and reducing sugars were carried out by adopting the classical procedures of Dubois *et al.* (1956) and Somogyi (1952). Soluble protein content was estimated following the protein-dye binding method (Bradford, 1976). The *in vivo* invertases and proteases were assayed by the methods of Jaynes and Nelson (1971) and Basha and Beevers (1975), respectively. For the study of *in vivo* kinetics of phosphatases, the sample extracts were incubated for 30 min in 0.01 M disodiumphenylphosphate (Fishmann and Davidson, 1975) and the component acid phosphatases and alkaline phosphatases were determined.

The experiment was repeated five times and the statistical analysis of data was done as ANOVA (Sokal and Rohlf 1973).

RESULTS AND DISCUSSION

Azadirachta indica A. Juss. (family Meliaceae) has a single annual growth flush with corresponding four periods, pre-anthesis/bud sprout, anthesis/flower bloom, post-anthesis/fruit set and fruit senescence/seed maturation. The annual regenerative phase initiated in March is marked with vegetative rejuvenation and terminates with fruit drop in July monsoon.

Reproductive development: The number of bud sprouts/ flowers per inflorescence was higher in test branches as compared to control. 0.5 mM kinetin induced significant bud burst, resulting in the development of more flowers. Post-anthesis, both 0.2 mM and 0.5 mM kinetin recorded enhanced fruit set. This was in contrast to 0.2 mM and 0.5 mM GA_3 treatments which did not induce budding. 0.5 mM GA_3 treatment was considerably better than the untreated control in inducing the reproductive ontogenic process and promoted on flowering and fruit development more as compared to the treatments with 0.2 mM GA_3.

Application of kinetin and GA_3 induce processes integral to fruit development (Palni *et al.* 1990, Jacqmard *et al.* 1994). Exogenously applied hormones promoted fruit set, increased fruit size, flower bloom and growth of developing fruit. An increased growth regulator supply during early fruit growth (the period of cell division) expresses a large increase in size as well as in quality with enhanced dry matter. However, in certain cases (GA_3 0.2 mM and kinetin 0.2 mM) no increase in fruit size was observed despite the treatments. This appears to be due to extra fruit set by flower that otherwise get abscised later on (Angrish and Dhir, 1996).

Total Sugars: Exogenous sprays of 0.5 mM and 0.2 mM kinetin effectively enhanced total sugars. From bud initiation upto anthesis sugar titers were significantly high (S_1 and S_2). Subsequently, during fruit development (S_3), total sugars rose with the respective treatments. A decline in sugar levels coincided with fruit senescence (S_4). 0.5 mM kinetin treatments promoted sugar levels in comparison to its counterparts at senescence. 0.2 mM GA_3 did not significantly promote sugar levels at the given stages, however, GA_3 0.5 mM was significantly

Table 1. Exogenous effect of growth regulators during reproductive growth flush

Treatments	Proteinsmg/g fr.wt.				Total Sugarsmg/g fr.wt.				Reducing Sugarsmg/g fr.wt				Invertaseμg/mg prot.			
	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
GA0.5 mM	272.0± 0.25	247.0± 0.16	285.0± 0.17	228.0± 0.28	634.0± 0.15	450.0± 0.15	660.0± 0.26	390.0± 0.36	341.0± 0.15	283.0± 0.40	425.0± 0.15	214.0± 0.35	248.0± 0.20	234.0± 0.26	260.0± 0.26	220.0± 0.15
GA0.2 mM	266.0± 0.15	240.0± 0.34	282.0± 0.20	220.0± 0.45	612.0± 0.11	439.0± 0.28	630.0± 0.52	364.0± 0.11	319.0± 0.23	270.0± 0.14	412.0± 0.10	202.0± 0.25	243.0± 0.11	230.0± 0.15	251.0± 0.10	211.0± 0.20
Kinetin 0.5 mM	320.0± 0.28	295.0± 0.20	325.0± 0.13	265.0± 0.30	719.0± 0.35	486.0± 0.26	740.0± 0.75	430.0± 0.58	411.0± 0.35	335.0± 0.26	496.0± 0.20	272.0± 0.43	299.0± 0.20	276.0± 0.58	310.0± 0.20	255.0± 0.20
Kinetin0.2 mM	313.0± 0.17	290.0± 0.57	307.0± 0.55	256.0± 0.30	695.0± 0.73	477.0± 0.11	720.0± 0.23	403.0± 0.15	394.0± 0.17	321.0± 0.55	490.0± 0.34	258.0± 0.30	294.0± 0.20	272.0± 0.17	304.0± 0.72	245.0± 0.30
Control	255.0± 0.36	230.0± 0.23	295.0± 0.12	210.0± 0.55	600.0± 0.86	490.0± 0.11	52.0± 0.13	370.0± 0.37	235.0± 0.23	254.0± 0.49	404.0± 0.52	195.0± 0.20	245.0± 0.69	230.0± 0.23	286.0± 0.96	200.0± 0.25
C.D.(at 5%)	0.47	0.36	0.35	0.99	0.67	0.53	0.69	0.61	0.45	0.88	0.54	0.66	0.63	0.52	0.33	0.38

over untreated control. In conformity to the results with 0.5 mM kinetin, 0.5 mM GA_3 samples sustained higher sugars over control during senescence (S_4).

Reducing sugars were enhanced considerably by 0.2 mM and 0.5 mM kinetin treatments in all the reproductive stages as compared to control and other treatments. Values (mg g^{-1} fr. wt.) peaked during fruit development for all treatments (S_3). 0.5 mM GA_3 recorded excess reducing sugars over other treatments at senescence which was significant as compared to control (S_4). Total extractable reducing sugars remained insignificant in 0.2 mM GA_3.

Invertase: 0.2 mM and 0.5 mM kinetin treatments were significant at budding (S_1). At flower bloom, 0.5 mM kinetin samples recorded higher invertase activity (µg mg^{-1} protein) over 0.2 mM kinetin samples (S_2). The trend was conformed at post-anthesis (S_3) when 0.5 mM kinetin treatment was higher as compared to 0.2 mM kinetin test analyses. GA_3 at 0.5 mM developed significant counts. Decreased activity was recorded with 0.2 mM GA_3 in all stages. While terminating the experiment at day 80 (S_4), the highest activity was observed in 0.5 mM and 0.2 mM kinetin samples, which was significantly more than GA_3 treated samples and also, untreated controls.

Previously reported results on increased hormonal levels during regeneration (Bernier *et al.* 1990, Lejeune *et al.* 1994, Koul and Wahab 2007), point to their putative role in floral evocation-transition phase. Therefore, reproductive growth (floral buds to fruit set) is characterised by enhanced growth regulator stimulated activity, in contrast to untreated shoot apices which showed comparatively slow organogenesis and associated metabolism. Furthermore, the differences in distribution of metabolites at reproductive levels may be indicative of their specific physiological role in re-mobilisation during regenerative processes.

Soluble Proteins: At pre-anthesis budding (S_1), 0.2 mM and 0.5 mM kinetin recorded higher protein values (mg g^{-1} fr.wt.) with respect to the annual sprays of GA_3. The concomitant soluble protein levels decreased with anthesis (S_2). Fruit development (S_3) featured a second peak in proteins with highest levels in 0.5 mM kinetin samples. Even untreated control increased unit protein at this stage. However, during fruit ripening, protein turnover was observed to be relatively slow (S_4). Soluble proteins at senescence were recorded best in kinetin 0.2 mM and 0.5 mM treatments. These values were significant when compared to GA_3 over untreated control at fruit senescence.

Protease: With relatively low activity µg mg^{-1} protein during budding (S_1), test analyses were significant at anthesis (S_2) – highest values were recorded with kinetin test samples. During fruit development, we evaluated the concomitant protease distinctly in 0.5 mM kinetin test samples (S_3). This was significantly higher than 0.5 mM GA_3 applications. During fruit senescence, 0.5 mM and 0.2 mM kinetin treatments had overtaken all other annual treatments with enzyme activity observed to be significantly over control (S_4).

Lejeune *et al.* (1994) suggested that the growth regulators may be part of a multi-component floral stimulus that induce a mitotic wave and some other events normally associated with flowering. Hence, they may act as the transmissible signal triggering cells of meristem into mitosis during early floral transition. Altered hormonal concentration before and after flower induction was reported for some species (Day *et al.* 1995, Dewittee *et al.* 1999). Our studies demonstrate that the effect of kinetin and GA_3 on regenerative process is dependent on the developmental stage of apical meristem (budding to fruit ripening). Towards the end of vegetative phase, hormonal- based signals tend to diminish as was also reported by Lejeune *et al.* (1994) in *Sinapis alba*. This was reflected in the untreated controls characterised by growth arrest and abscission. Exogenous kinetin promoted reproductive organogenesis as well as fruit/ seed set in our studies. The decreased organogenesis during this phase in untreated samples coincided with probably low endogenous hormonal levels in the meristems. With exogenous supply, sprouting buds got activated earlier resulting in the growth of floral organs. During further development, all biomolecules as well as enzymes were observed to be strongly influenced. This might be indicative of a better sink metabolic activity of reproductive areas.

Table 2. Exogenous effect of growth regulators during reproductive growth flush

Treatments	Proteasemg/mg prot.				Acid Phosphatasemg/mg prot.				Alkaline Phosphatasemg/mg prot.				Per Panicle			
	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4	Buds	Flowers	Fruit set	Seed set
GA0.5 mM	98.0± 0.15	83.0± 0.17	103.0± 0.58	88.0± 0.55	466.0± 0.98	405.0± 0.37	482.0± 0.25	369.0± 0.28	398.0± 0.34	346.0± 0.25	375.0± 0.10	185.0± 0.15	126.0± 1.54	121.0± 2.71	120.0± 1.76	118.0± 2.27
GA0.2 mM	94.0± 0.98	81.0± 0.10	99.0± 0.58	89.0± 0.30	451.0± 0.20	400.0± 0.80	470.0± 0.20	364.0± 0.15	265.0± 0.47	340.0± 0.30	367.0± 0.15	181.0± 0.45	122.0± 2.74	118.0± 3.09	116.0± 3.07	115.0± 2.09
Kinetin 0.5 mM	121.0± 0.35	97.0± 0.73	126.0± 0.75	96.0± 0.23	540.0± 0.35	470.0± 0.11	530.0± 0.78	471.0± 0.12	454.0± 0.15	413.0± 0.25	449.0± 0.10	247.0± 0.12	118.0± 2.91	116.0± 1.97	113.0± 1.01	110.0± 2.11
Kinetin 0.2 mM	108.0± 0.20	91.0± 0.50	119.0± 0.60	92.0± 0.63	514.0± 0.40	446.0± 0.15	517.0± 0.60	449.0± 0.15	436.0± 0.14	398.0± 0.90	430.0± 0.21	233.0± 0.24	116.0± 1.48	118.0± 2.08	113.0± 1.71	112.0± 3.09
Control	98.0± 0.36	82.0± 0.12	106.0± 0.63	90.0± 0.13	440.0± 0.72	404.0± 0.75	390.0± 0.78	360.0± 0.12	345.0± 0.10	340.0± 0.23	380.0± 0.10	177.0± 0.19	104.0± 1.32	112.0± 1.05	110.0± 1.32	96.0± 1.73
C.D.(at 5%)	0.79	0.49	0.22	0.38	0.66	0.55	0.85	0.23	0.99	0.87	0.54	0.58	7.59	1.42	7.92	5.16

Phosphatases: Generally acid phosphatase activity remained greater than alkaline phosphatase throughout our experimental analyses (µg mg^{-1} protein) and also increased significantly relative to control samples in the reproductive stages. The initial exogenous sprays enhanced acid phosphatase with respect to kinetin treatments (S_1). The trend following anthesis (S_2) regained with 0.5 mM as well as 0.2 mM kinetin which were significantly better over GA_3 treatments even in reference to untreated control. While reaching post-anthesis, the most significant activity was recorded with samples of 0.5 mM kinetin / 0.5 mM GA_3 (S_3). At final reproductive stage, 0.5 mM kinetin / 0.2 mM kinetin showed peak activities, greater than all the previous observations (S_4) and over control.

Kinetin (0.5 mM) induced fruit set and yield. *In vivo* treatments enhanced the numbers of flowers per panicle and the fruit set post-anthesis. Biochemical estimations constituting total sugars, reducing sugars, proteins and related enzymes also improved. Application of GA_3 did not yield similar results; this suggests that the plant growth regulator had probably reached its saturation mark in the "metabolic pool of reproductive morphogenesis" (Palni *et al.* 1990, Binns, 1994, Chaturvedi *et al.* 2003). The values of parameters like number (buds, flowers, fruit and seed set) and quality (proteins, sugars and enzyme levels) correlated with the measure of yield decline in the GA_3 samples. Therefore, the maximum potential to increase yield in Neem is achieved with kinetin application, the excess GA_3 tended to negate its own effect as part of the negative feedback mechanism discussed by Palni *et al.* (1990) and Binns (1994).

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