NEEM: *AZADIRACHTA INDICA* A. JUSS. REPRODUCTIVE STUDIES IN RELATION TO POLYAMINE APPLICATION

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SUMMARY

The *in vivo* morpho-physiological responses to polyamine application in Neem (*Azadirachta indica*) were studied during three consecutive annual reproductive flushes from bud development onwards to seed set (prior standardization with endogenous levels). The number of buds and flowers per inflorescence was higher in treated branches as compared to the control. Physiological responses were observed to be polyamine-specific. Sample protein, total sugar and reducing sugar measured higher following spermidine and putrescene as compared to spermine. The response was significant in 0.2 mM spermidine samples. Spermine treatments produced poor flowering response, longer juvenility and low contents of the above compounds as compared to the control. The enzyme assay measuring the activities of invertase, protease, acid- and alkaline phosphatases was consistent with the biochemical responses to the polyamine treatments. In control, a shorter regeneration phase seems to lead to lower productivity in terms of floral inflorescence and fruit set. Therefore, it is suggested that the application of spermidine and putrescene may regulate the reproductive phase resulting in improved organ/biomolecule production.

***Key words*: *Azadirachta indica*, neem, putrescene, reproduction, spermidine, spermine.**

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, Koudal *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 (Hansen *et al.* 1996, Walia *et al*. 2002, Nathan *et al.* 2006, Koul and Wahab 2007).

In recent years, the foliar application of plant growth regulators on cash crops has enhanced plant growth, development and yield (Abd El-Wahed and Gamal El-Din 2004, Vila *et al.* 2004, Abd El-Wahed 2006). Spermidine and spermine (a low molecular weight, aliphatic, triamine and tetramer, respectively) and its precursor compound, putrescene have now been recognized as plant growth regulators which can mediate reproductive differentiation in various plants (Martin-Tanguy 2001, Aziz 2003). These reports are based on their ubiquity, abundance in growing tissues and their effects on growth and development (Angeles Botella 2000, Koul and Wahab 2007). In addition to various responses produced at the molecular level, polyamines influence protein, nucleic acid and mitotic activity (Behl *et al.* 2000, Wallace *et al.* 2003) and also play a role in cell division, growth and induction of invertase and phosphatase syntheses (Pandey *et al.* 2000, Vila *et al.*

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2003). They also regulate the promotive effect of gibberellins and cytokinins (Pandey *et al.* 2000, Sharry *et al.* 2006).

This information indicates that polyamines are akin to other established plant growth stimulants in the regulation of cellular metabolic activity. However, most of the information is restricted to the vegetative phase of a plant and there is little information in respect to reproductive phase, especially on fruit. The present study investigates the effect of putrescene, spermidine and spermine, respectively on some biochemical parameters during reproductive growth of Neem.

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 onset of the regeneration phase in March, the apices of the test branches (in replicates of five) were sprayed *in vivo* with standardized putrescene, spermidine and spermine solutions, respectively and a few drops of Tween 20. The control branches were tagged.

Preliminary experimentation: In accordance to previous test analyses and studies (Sokal and Rohlf 1973, Devkumar and Sukhdev 1993), the polyamines were applied over a concentration range of 0.1 mM – 3.0 mM in steps of 0.2 mM. Certain parameters such as number of reproductive structures per panicle, their size, weight and quality were recorded and the best treatments were correlated and standardized (Sabherwal 2000).

Main experimentation: The treatments selected for final study were 0.5 mM and 0.2 mM of the three polyamines applied in separate test branches (in replicates of five) in three consecutive years. Observations were recorded separately at various stages of reproductive development, such as bud sprout (pre-anthesis), flower bloom (anthesis), fruit set (post-anthesis) and seed (senescence). 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). RNA content was determined by the modified orcinol method at a wavelength of 610.0 nm on Shimadzu double beam spectrophotometer (Nieman and Poulson 1963). The *in vivo* invertases and proteases were assayed by the methods of Jaynes and Nelson (1971) and Basha and Beevers (1975), respectively. Samples of reproductive tissue from each treatment were collected at 20, 40, 60 and 80 days after the initial spray. For the study of *in vivo* kinetics of phosphatases, sample extracts from control branches and of those sprayed with the optimal concentrations of the polyamines were incubated for 30 min in 0.01 M disodiumphenylphosphate (Fishmann and Davidson 1975) and the component acid phosphatases and alkaline phosphatases were determined : 1) acetate buffer pH 5.2, 2) carbonate-bicarbonate buffer pH 10, respectively.

The statistical analyses of data was done by ANOVA tests available in software packages as outlined by Sokal and Rohlf (1973).

RESULTS AND DISCUSSION

Azadirachta indica A. Juss. (Family Meliaceae) has a single annual growth flush with four corresponding periods; pre-anthesis (bud sprout), anthesis (flower bloom), post-anthesis (fruit set) and senescence (seed). The annual regenerative phase initiates in March marked with vegetative rejuvenation; and terminates with fruit drop in July, which coincides with the monsoons and strong breeze. The ripe fruit may be subject to microorganism and insect activity, feeding birds and human harvest.

Reproductive development : The number of bud sprouts/flowers per inflorescence was higher in test branches as compared to control. 0.2 mM polyamines induced significant bud burst (Table 1), resulting in the development of more flowers (Table 2). Post-anthesis (Table 3), both 0.2 mM and 0.5 mM polyamines recorded enhanced fruit set. A favorable response with 0.2 mM spermidine improved reproductive structures, the number being significantly greater than the control. This was in contrast to 0.5 mM spermine treatment which did not induce budding. 0.5 mM putrescene treatment,

Table 1. Exogenous effect of Polyamines during reproductive growth flush – Metabolites at Stage I Pre-anthesis

Treatments	Proteins mg/g fr.wt.	Total sugars mg/g fr.wt.	Reducing sugars mg/g fr.wt.	DNA µg/g dry wt.	RNA µg/g dry wt.	Invertase µg/mg prot.	Protease µg/mg prot.	Acid Phos-phatase µg/mg prot.	Alkaline phosphatase µg/mg prot.	No. of buds/ panicle
Put1 (0.2)	307.0±0.17	651.0±0.58	395.0±0.58	176.0±0.10	237.0±0.15	287.0±0.15	109.0±0.15	489.0±0.57	443.0±0.20	127.0±1.59
Put2 (0.5)	302.0±0.36	636.0±0.52	385.0±0.58	174.0±0.26	228.0±0.20	279.0±0.23	106.0±0.75	469.0±0.57	410.0±0.17	124.0±2.11
Spd1 (0.2)	331.0±0.20	758.0±0.20	440.0±0.25	214.0±0.25	310.0±0.75	305.0±0.15	126.0±0.26	582.0±0.14	416.0±0.20	131.0±1.30
Spd2 (0.5)	325.0±0.60	736.0±0.28	408.0±0.34	210.0±0.20	331.0±0.58	301.0±0.11	122.0±0.58	565.0±0.17	456.0±0.58	128.0±1.00
Spm1 (0.2)	276.0±0.57	680.0±0.26	370.0±0.37	166.0±0.46	214.0±0.26	263.0±0.10	103.0±0.13	496.0±0.34	353.0±0.69	126.0±1.49
Spm2 (0.5)	283.0±0.98	669.0±0.30	350.0±0.69	164.0±0.25	199.0±0.17	253.0±0.11	100.0±0.17	479.0±0.17	289.0±0.25	122.0±2.07
Control	260.0±0.55	619.0±0.37	293.0±0.20	160.0±0.15	204.0±0.52	255.0±0.25	103.0±0.13	454.0±0.12	353.0±0.19	112.0±1.73
C.D. (at 5%)	0.47	0.67	0.45	0.39	0.72	0.63	0.79	0.69	0.99	7.59

though considerably better than the untreated control in inducing the reproductive ontogenic process, was less promotory on flowering and fruit development as compared to the treatments with 0.2 mM putrescene (Table 4).

Total Sugars : Exogenous sprays of 0.2 mM spermidine and putrescene effectively enhanced total sugars. From bud initiation up to anthesis sugar titers were significantly high (Tables 1 and 2). Subsequently during fruit development (Table 3), total sugars rose with the respective treatments. A decline in sugar levels coincided with fruit senescence (Table 4). 0.2 mM spermidine treatments promoted sugar levels in comparison to its counterparts at senescence. Spermine did not significantly promote sugar levels at the given stages, however, the treatments were significant over control. In conformity to the results with 0.2 mM polyamines, 0.5 mM polyamine samples sustained higher sugars over control during senescence (Table 4).

Reducing sugars were enhanced considerably by 0.2 mM and 0.5 mM putrescene 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 (Table 3). 0.2 mM

Table 2. Exogenous effect of Polyamines during reproductive growth flush – Metabolites at Stage II Anthesis

Treatments	Proteins mg/g fr.wt.	Total sugars mg/g fr.wt.	Reducing sugars mg/g fr.wt.	DNA µg/g dry wt.	RNA µg/g dry wt.	Invertase µg/mg prot.	Protease µg/mg prot.	Acid Phos-phatase µg/mg prot.	Alkaline phosphatase µg/mg prot.	No. of buds/ panicle
Put1 (0.2)	282.0±0.20	476.0±0.11	327.0±0.20	170.0±0.49	207.0±0.25	270.0±0.58	89.0±0.26	435.0±0.13	392.0±0.26	128.0±1.22
Put2 (0.5)	277.0±0.14	448.0±0.13	312.0±0.49	159.0±0.58	198.0±0.40	260.0±0.58	87.0±0.11	414.0±0.26	390.0±0.58	126.0±1.04
Spd1 (0.2)	303.0±0.15	542.0±0.40	369.0±0.10	202.0±0.10	263.0±0.15	290.0±0.58	108.0±0.40	510.0±0.57	435.0±0.58	138.0±1.79
Spd2 (0.5)	292.0±0.15	504.0±0.30	375.0±0.40	196.0±0.23	251.0±0.50	285.0±0.58	101.0±0.25	498.0±0.57	419.0±0.15	130.0±1.08
Spm1 (0.2)	259.0±0.63	448.0±0.32	310.0±0.20	148.0±0.28	196.0±0.40	244.0±0.10	87.0±0.58	419.0±0.10	381.0±0.58	125.0±1.81
Spm2 (0.5)	250.0±0.15	438.0±0.46	299.0±0.20	135.0±0.25	174.0±0.15	238.0±0.11	85.0±0.58	409.0±0.20	366.0±0.23	121.0±2.14
Control	237.0±0.12	506.0±0.13	364.0±0.52	137.0±0.89	184.0±0.26	240.0±0.96	89.0±0.63	414.0±0.78	348.0±0.10	125.0±1.32
C.D. (at 5%)	0.36	0.53	0.88	0.48	0.52	0.52	0.49	0.55	0.87	1.42

Table 3. Exogenous effect of Polyamines during reproductive growth flush – Metabolites at Stage III Post-anthesis

Treatments	Proteins mg/g fr.wt.	Total sugars mg/g fr.wt.	Reducing sugars mg/g fr.wt.	DNA µg/g dry wt.	RNA µg/g dry wt.	Invertase µg/mg prot.	Protease µg/mg prot.	Acid Phos-phatase µg/mg prot.	Alkaline phosphatase µg/mg prot.	No. of buds/ panicle
Put1 (0.2)	321.0±0.11	676.0±0.58	501.0±0.12	194.0±0.25	252.0±0.15	298.0±0.19	136.0±0.58	512.0±0.57	425.0±0.58	123.0±1.45
Put2 (0.5)	314.0±0.25	660.0±0.11	478.0±0.20	191.0±0.28	242.0±0.20	290.0±0.11	130.0±0.34	489.0±0.45	413.0±0.65	123.0±1.31
Spd1 (0.2)	344.0±0.55	780.0±0.52	530.0±0.35	220.0±0.20	256.0±0.36	316.0±0.57	131.0±0.35	561.0±0.10	475.0±0.23	129.0±1.07
Spd2 (0.5)	336.0±0.15	760.0±0.80	510.0±0.26	216.0±0.20	247.0±0.30	311.0±0.58	127.0±0.36	551.0±0.11	455.0±0.28	127.0±2.06
Spm1 (0.2)	288.0±0.41	700.0±0.25	469.0±0.20	176.0±0.15	229.0±0.20	274.0±0.15	109.0±0.23	571.0±0.15	402.0±0.19	121.0±2.11
Spm2 (0.5)	296.0±0.16	693.0±0.26	447.0±0.58	173.0±0.28	217.0±0.26	268.0±0.10	106.0±0.17	498.0±0.17	460.0±0.35	117.0±2.34
Control	303.0±0.23	534.0±0.11	404.0±0.49	203.0±0.58	227.0±0.36	276.0±0.23	109.0±0.12	506.0±0.75	386.0±0.23	122.0±1.05
C.D. (at 5%)	0.35	0.69	0.54	0.57	0.43	0.33	0.22	0.85	0.54	7.92

spermidine recorded excess reducing sugars over other treatments at senescence which was significant as compared to control (Table 4). Total extractable reducing sugars remained insignificant in spermine.

Invertase : 0.2 mM and 0.5 mM polyamines were significant at budding (Table 1). At flower bloom, 0.2 mM samples recorded higher invertase activity (µg mg^{-1} protein) over 0.5 mM samples (Table 2). The trend was conformed at post-anthesis (Table 3) when 0.2 mM polyamine treatment was high in test analyses. Spermidine and putrescene at 0.2 mM developed significant counts. Decreased activity was recorded with 0.5 mM spermine in all stages (Tables 1-4). While terminating the experiment at day 80 (Table 4), the highest activity was observed in 0.2 mM and 0.5 mM spermidine samples, which was significantly more than the spermine treated samples and also, untreated controls.

Cases of polyamine-induced fertility have been reported previously in several plants (Dhir *et al.* 1986, Angrish and Dhir 1996, Martin-Tanguy 2001). Developmental periods within the reproductive phase, such as bud initiation, floral bloom, fruit growth and

Table 4. Exogenous effect of Polyamines during reproductive growth flush – Metabolites at Stage IV Fruit senescence

Treatments	Proteins mg/g fr.wt.	Total sugars mg/g fr.wt.	Reducing sugars mg/g fr.wt.	DNA µg/g dry wt.	RNA µg/g dry wt.	Invertase µg/mg prot.	Protease µg/mg prot.	Acid Phos-phatase µg/mg prot.	Alkaline phosphatase µg/mg prot.	No. of buds/ panicle
Put1 (0.2)	252.0±0.11	432.0±0.15	263.0±0.25	128.0±0.15	178.0±0.26	243.0±0.11	107.0±0.11	410.0±0.57	227.0±0.19	124.0±1.41
Put2 (0.5)	244.0±0.40	420.0±0.41	251.0±0.11	125.0±0.24	173.0±0.10	238.0±0.58	102.0±0.58	385.0±0.24	214.0±0.10	121.0±1.22
Spd1 (0.2)	276.0±0.30	473.0±0.58	307.0±0.72	145.0±0.58	229.0±0.20	270.0±0.11	103.0±0.25	499.0±0.20	227.0±0.15	127.0±1.00
Spd2 (0.5)	274.0±0.36	450.0±0.15	282.0±0.10	137.0±0.28	213.0±0.28	261.0±0.15	118.0±0.32	480.0±0.49	258.0±0.17	123.0±2.11
Spm1 (0.2)	235.0±0.43	396.0±0.89	242.0±0.43	126.0±0.58	160.0±0.75	234.0±0.25	98.0±0.30	385.0±0.37	203.0±0.58	120.0±1.46
Spm2 (0.5)	229.0±0.23	381.0±0.26	223.0±0.11	123.0±0.45	147.0±0.13	229.0±0.23	94.0±0.30	378.0±0.15	194.0±0.20	118.0±1.28
Control	221.0±0.36	392.0±0.86	203.0±0.23	121.0±0.52	144.0±0.58	207.0±0.69	95.0±0.36	370.0±0.72	187.0±0.10	110.0±1.32
C.D. (at 5%)	0.99	0.61	0.66	0.64	0.45	0.38	0.38	0.23	0.58	5.17

maturity were affected positively by polyamines treatment. In control branches only a low average number of flowers produced fruit, while in the polyamine-treated branches, there was a higher average number of fruit at post-anthesis, some of which abscised during ripening. The number of fruit set increased with 0.2 mM spermidine applications. Spermidine at 0.5 mM also promoted fruit development. An improvement in the quality of fruit with putrescene treatment was apparent in terms of increased total sugars and invertase activity. Fruit matured in 22-27 days following which senescence resulted in fruit abscission. In developing tissues, polyamines probably accelerated the conversion of insoluble carbohydrates to soluble sugars (Aziz 2003). Reducing sugar content was also increased.

Soluble Proteins : At pre-anthesis budding (Table 1), treatments recorded higher protein values (mg g^{-1} fr. wt.) with respect to the other annual stages. The concomitant soluble protein levels decreased with anthesis (Table 2). Fruit development (Table 3) featured a second peak in proteins with highest levels in 0.2 mM spermidine followed by 0.2 mM putrescene samples. Even the spermine treatments increased unit protein at this stage. However, during fruit ripening, protein turnover was observed to be relatively slow (Table 4). Soluble proteins at senescence were recorded best in spermidine and putrescene 0.2 mM and 0.5 mM treatments. These values were significant when compared to spermine treatments over untreated control at fruit senescence.

Protease : With relatively low activity μg mg^{-1} protein during budding (Table 1), test analyses were significant at anthesis (Table 2) – highest values were recorded with spermidine samples. During fruit development, we evaluated the concomitant protease distinctly in 0.2 mM putrescene test samples (Table 3). This was significantly higher than 0.5 mM putrescene applications. By fruit senescence, 0.2 mM and 0.5 mM spermidine treatments had overtaken all other annual treatments with enzyme activity observed to be significant over control (Table 4).

The *in vivo* protease activity was directly proportional to soluble protein, its substrate, in both untreated branches and those treated with growth regulators. Protease, determined in the pharmaceutically important fruit-set (Devakumar and Sukhdev 1993, Aziz 2003), ranged between 106.0 – 109.0 units in untreated control and spermine treatments, but increased significantly to 130 – 136 units in putrescene and to 127 – 131 units in spermidine applications. As protease μg mg^{-1} protein remained nearly the same in untreated samples and spermine treatments, there seems to be little alteration in the number of active sites in the oxido-reductive protein (Martin-Tanguy 2001). Conversely, the catalytic property remains unaffected with the application of these biomolecules. A possible explanation for the increased enzyme activity in case of spermidine is the enhanced syntheses of protein and proteolysis. This observation can be deduced from studies which suggested that different effect of NH_4^+ and NO_3 on growth may be partly due to high polyamine levels (Abd El-Wahed 2006).

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 during pre-anthesis enhanced acid phosphatase with respect to the polyamine treatments (Table 1). Subsequent annual sampling steadily depreciated enzyme counts at post-anthesis. The trend following anthesis (Table 2) re-gained with spermidine treatments which were significantly better over putrescene treatments even in reference to untreated control. While reaching post-anthesis, the most significant activity was recorded with samples of 0.2 mM spermidine / 0.2 mM putrescene (Table 3). At final reproductive stage, 0.2 mM spermidine / 0.5 mM spermidine showed peak activities, even greater than all other observations set forth in the respective treatments (Table 4) and over control.

Phosphatases have a role in phosphorus metabolism during the early phase of reproductive transformation (Chaturvedi *et al.* 2003). In our study, high activity of phosphatases during periods of active growth / differentiation suggest a positive correlation between phosphatase and reproductive development. Phosphatases activity was associated with pre-anthesis (Table 1) and post-anthesis (Table 3). Enzyme activity

dropped during anthesis (Table 2) as well as fruit senescence (Table 4). Apparently, phosphatases, along with invertase and protease increased active metabolism — their activity supported by translocation of assimilates; total sugar, reducing sugar and soluble proteins to the growing organs of regeneration (Sharry *et al.* 2006). In this respect, Durdan *et al.* (2000) proposed the nutrient diversion theory, which explains flower induction as modifying source / sink relationships within the plant, leading to higher concentration of assimilates in the shoot apex under the inductive regime.

Exogenous applications of spermidine and putrescene, respectively were related to flowering, rapid growth and high metabolic activity in our study. Polyamines were associated with floral induction and floral development in previous studies (Abd El-Wahed and Gamal El-Din 2004). Polyamine translocation from leaves following photoperiodic flowering induction was envisaged (Havelange *et al.* 1996, Vila *et al.* 2004). Pandey *et al.* (2000) suggested that an increased supply of polyamines leads to a general depression of ethylene senescence system. Enhanced biochemical and enzymatic activities in our study are also indicative of the observation made by Aziz (2003). Spermidine at 0.2 mM, induced the best fruit set and yield with the annual sprays. *In vivo* treatments improved the numbers of flowers/panicle and the fruit set post-anthesis. However, the effectiveness was greatly reduced in spermine treatments. Biochemical estimations constituting total sugar, reducing sugar, protein and the related enzymes declined. This suggested that the foliar application of the plant growth regulator on Neem to enhance plant growth, development and yield had probably no significant function in the "metabolic pool of reproductive morphogenesis" (Wallace *et al*. 2003, Abd El-Wahed 2006).

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