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AZADIRACHTA INDICA A. JUSS. (NEEM) : SEED OIL & LIMONOIDS RELATED TO GROWTH REGULATOR TREATMENTS

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Endogenous titres of "neemrich' was stimulated with exogenously applied hormones. In our efforts to study and control the commercial seed yield harvest, results distinctly indicated the regulatory influence of hormone combinations: kinetin-GA_3 and kinetin-spermidine in maintaining higher limonin levels during maturation relative to control samples. Variations in limonoids and seed oil was observed during the five classes of growth regulator applications, *i.e.*, putrescine, spermidine, spermine, GA_3 and kinetin. Limonoid content was generally high in samples treated with kinetin 0.2 mM and spermidine 0.2 mM. The levels of seed oil increased in 0.5 mM concentration treatments in spermine, putrescine and spermidine. The high concentrations of polyamines proved inhibitory to limonin levels and promotory to seed oil content. A steady rise in limonin was noted with GA_3 while decreasing the exogenous supply from 0.5 mM to 0.2 mM levels. Thus, the results are of potential pharmaceutical significance in terms of understanding and enhancing regeneration and seed yield.

KEY WORDS: *Azadirachta indica*, GA_3, kinetin, limonoids, neem, polyamines, seed oil.

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 may, in fact, be the source of a new generation of chemicals for use in Integrated Pest Management (Walia *et al*. 2002, Koul and Wahab 2007).

Previous studies indicate the involvement of certain growth regulators during regenerative growth flush (Tamas 1987, Dewittee *et al.* 1999, Angeles Botella *et al.* 2000). 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). Kinetin and GA_3 have now been recognized as plant growth regulators which can mediate reproductive differentiation in various plants (Dhir *et al.* 1986, Letham 1994, Metzger 1995, Koul and Wahab 2007). These reports are based on their ubiquity, abundance in growing tissues and their effects on growth and development (Martineau *et al.* 1995, Kaminek *et al.* 1997, Durdan *et al.* 2000). Polyamines (putrescine, spermidine and spermine) have also been recognized as plant growth regulators which in addition to various responses produced at molecular level; influence protein, nucleic acid and mitotic activity (Martin-Tanguy 2001, Aziz 2003) and also play a role in cell division, growth and induction of invertase and phosphatase synthesis (Pandey *et al.* 2000, Vila *et al.* 2004). Considering the role of plant growth regulators in regeneration and development, an experiment was designed as an extension to our previous work (Sabherwal and Dhir 2009,10). The principle aims of this study are: (i) to determine the regulatory role of exogenously applied growth regulators on the active principles in seed, (ii) to assess the role of the plant growth substance in the secondary metabolic pathway of biologically active compounds and (iii) to exploit the potential of growth regulator in increasing the pharmaceutical yield.

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This was taken as a significant research problem since the product of the reproductive phase, that is, the neem seed; is commercially important in Integrated Pest Management and pharmaceuticals. Moreover, neem is native to the Indian sub-continent; and inferences from previous work on some other plants indicate that there may be a definite possibility to enhance the yield.

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, apices of the test branches were sprayed *in vivo* with standardized kinetin, polyamine and GA_3 solutions with a few drops of Tween 20.

Preliminary experimentation: In accordance to previous test analysis and studies (Sokal and Rohlf 1973, Devakumar 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 standardized (Sabherwal and Dhir 2009, 10).

Main experimentation: In the present study individual treatments selected were putrescine, spermidine, spermine, kinetin and GA_3 applied in concentration 0.2 mM and 0.5 mM, respectively. Also 0.5 mM kinetin was applied in combinations with spermidine 0.2 mM, 0.5 mM and GA_3 0.5 mM to separate test branches (in replicates of five) in three consecutive years. Free limonoids tetranortriterpenoids and fatty oil content were determined by the method of Devakumar and Sukhdev (1993). In all the treatments, endogenous profiles (comprising 'neemrich') recorded were seed index, fatty oil content and limonoid for the effective pharmaceutical inference determined according to limonoid:seed index (l:I) and limonoid:fatty oil (l:f) (Table 1). After prior soxhlet extraction of the seed post-anthesis, the active principle in the samples were detected by Shimadzu HPLC using ODS chromosorb column (250.0 mm x 4.6 mm) with a mobile phase of methanol:water (60:40, v/v). Each run was set to 60 minutes. The calibration was as per the peak-area data of HPLC elutions with standards (Sigma chemicals and IARI, New Delhi).

The statistical analysis of data was done as ANOVA in software according to Sokal and Rohlf (1973).

RESULTS AND DISCUSSION

Azadirachta indica A. Juss. (family Meliaceae) has a single annual growth flush with budding and flower blooms in March developing to post-anthesis fruit set-seed. The ripe fruit set in July coincides with the heavy monsoons along with incessant insect attack, feeding birds and human harvest.

Limonoid evaluations : Neem seeds are normally sold by weight while their commercial value depends upon their mean limonoid content per unit weight (Govindachari *et al.* 1990, Devakumar and Sukhdev 1993). Therefore, our results were expressed as compound mg per unit g seed (Table 1). The 'neemrich' elution was influenced with growth regulator treatments indicating their possible role in secondary metabolism.

Fatty Oils (gram content per gram seed weight) and Seed Index (gram weight per 100 seeds) :

The variations in fatty oils (f) as well as seed index (I) among the hormone affected neem seed kernels are projected in Table 1. Untreated controls (C) as 0.49 units and 21.8

units, respectively remained the constant mark of comparison.

Thus, neem oils varied from a lower range 0.34-0.37 units with combination of hormones (kinetin-spermidine) as well as spermidine 0.2 mM, to a higher limit of 0.47-0.50 units with GA_3 0.5 mM and spermine treatments (Table 1). The fatty oils per seed recorded moderate levels with putrescine 0.2 mM: 0.45 units and putrescine 0.5 mM: 0.47 units as also kinetins: 0.43 units. The untreated samples (C) maintained substantial fatty acids (0.49 units). Therefore, exogenous growth regulators largely lowered the fat per seed content. This eventually had a diminutive effect upon seed index (mass per 100 seeds).

Much higher amounts of seed index (grams) as also, fatty oil content (grams), were respectively reported from spermine 0.5 mM (24.0/0.50 units), spermine 0.2 mM (22.2/0.48 units), GA_3 0.5 mM (20.0/0.47 units), putrescine 0.5 mM (21.3/0.47 units) and putrescine 0.2 mM (21.9/0.45 units). The relative changes evaluated in seed index were concomitant with oil contents in neem fruit as influenced by the growth regulator treatments (Table 1). Untreated controls (C) recorded seed index as 21.8 units. Low counts were recorded with hormone combinations as 16.4 units (kinetin 0.5 mM-GA_3 0.5 mM), 15.8 units (kinetin 0.5 mM-spermidine 0.2 mM) and 15.3 units (kinetin 0.5 mM-spermidine 0.5 mM). The individual growth regulators, spermidine 0.2 mM (15.0 units), kinetin 0.5 mM (18.9 units) and GA_3 0.2 mM (15.5 units), registered poor seed indices.

Application of kinetin and spermidine induced processes integral to fruit development (Palni *et al.* 1990, Jacqmard *et al.* 1994). Exogenously applied hormones promoted fruit set, increased fruit size and growth of developing fruit. An increased growth regulator supply during early fruit growth (the period of cell division) was expressed as large increase in size as well as quality with enhanced dry matter. However, in certain cases (GA_3 0.5 mM and kinetin 0.5 mM) no increase in fruit size was observed despite the treatments. This appears to be due to extra fruit set that otherwise get abscised later on (Angrish and Dhir 1996, Tucker 1996).

In our effort to commercially as well as pharmaceutically define the harvest seed yield, worthwhile ratios of seed production were obtained.

Mainly owing to its various effects on insects, limonoids are considered the most important active principle in neem seed kernels (Friend 1998). However, the quantity of this compound present in neem seed kernels vary considerably with exogenous growth regulator treatments; the hormone and its concentration. Higher yield of limonoids were obtained in tissues treated with putrescine, kinetin as well as GA_3. Limonoids are formed by a group of closely related alkaloids; nimbin, salannin and azadirachtins (Kraus *et al.*1985, Broughton *et al.* 1986). While azadirachtins are the most important compound in terms of its quantity in neem seed kernel extracts, all others are also effective insect growth regulators (Rembold 1990).

Total Limonoid content (gram compound per gram seed weight) : Limonoid content varied ranging from 0.003-0.006 units in spermine 0.5 mM, spermidine 0.5 mM as well as putrescine 0.5 mM test analyses as compared to control analyses (0.010 units). Hence, higher concentrations of all polyamine samples recorded poor 'neemrich' levels (Table 1). However, the content varied with certain growth regulators like kinetins (0.5 mM: 0.11 units, 0.2 mM: 0.12 units) and GA_3 (0.5 mM: 0.015 units, 0.2 mM: 0.054 units). Favourable

increase was observed with combination of hormones, kinetin 0.5 mM-GA_3 0.5 mM (0.091 units), kinetin 0.5 mM-spermidine 0.2 mM (0.020 units) and kinetin 0.5 mM-spermidine 0.5 mM (0.016 units). Indeed, the study revealed a significant correlative rise with respect to control samples (0.010 units). It was observed that low level GA_3 0.2 mM sprays (0.054 units) and /or alternatively; high level GA_3 0.5 mM in combination with kinetin 0.5 mM (0.091 units) considerably influenced the increase in limonoids. This suggested that the GA_3 had profound effect upon endogenous titres of 'neemrich' in commercial seed product. While the influence of polyamines was profound only in certain cases as spermidine 0.2 mM (0.021 units), kinetin 0.5 mM-spermidine 0.2 mM (0.020 units) and to some extent in spermine 0.2 mM (0.017 units), kinetin 0.5 mM-spermidine 0.5 mM (0.016 units) test samples.

In our efforts to commercially as well as pharmaceutically define the harvest seed yield, it seemed worthwhile to analyze ratios of seed production. Mean proportions of limonoids to fatty oils (l:f) as well as limonoids to seed index (l:I) were accordingly evaluated (Table 1).

Earlier, Yamasaki *et al.* (1986) isolated and purified 'neemrich' by normal-phase as well as reverse-phase HPLC, while Govindachari *et al.* (1990) purified the active principle on preparative HPLC and resolved as analytical HPLC with aqueous methanol. However, we found that the solvent on preparative HPLC improved the resolution.

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 fruit evocation-transition phase. Therefore, reproductive phase is characterized 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-mobilization during regenerative processes.

The active compounds isolated from neem seed kernels show anti-feedant activity in bio-test (Schwinger *et al*. 1984, Jones *et al*. 1990). Similar pesticidal properties are assigned to fatty oils (Kubo *et al*. 1986, Kraus *et al*. 1987).

Commercial Harvest Yield

Limonoid to Seed Index ratio (l:I) : Neem seed samples collected from thirteen treatment zones were analyzed for variation in seed index (I) and limonoid to seed index ratio (l:I) (Table 1). Seed index (gram weight per 100 seeds) was as low as 15.0 units for spermidine 0.2 mM collections and was valued between 15.3-19.7 units in kinetins and GA_3 0.2 mM. While the weight per 100 seeds (I) optimized between 21.3-24.0 units with putrescine as well as spermine, it was 21.8 units in untreated control samples (C). Conversely, limonoid to seed index ratio l:I was higher with GA_3 0.2 mM (0.210), kinetin 0.5 mM-GA_3 0.5 mM (0.33), spermidine 0.2 mM (0.084) as also, with kinetin 0.5 mM-spermidine 0.5 mM (0.077). The proportions were generally low in putrescine (0.2 mM: 0.036, 0.5 mM: 0.017) and spermine 0.5 mM treated samples with a registered drop at 0.007.

The data analyses revealed that seed index did not influence l:I value, and with increase/decrease in l:I, there was no marked increase/decrease in neem seed index. This also indicated that commercial yield product (limonoids) was not related to seed weight market purchase since there was no linear correlation between seed index and commercial harvest yield, denoted by l:I

values. Limonoids as well as seed index would, therefore, vary with the relative vigour and inherited variability in mother plant.

Limonoid to Fatty Oils ratio (l:f) : In the second set of commercial harvest yield inferences, limonoid utility was related to l:f evaluations. These have been briefly summarized in Table 1. Thus, l:f recorded beneficial proportions in hormone combinations kinetin 0.5 mM-GA_3 0.5 mM (0.213), kinetin 0.5 mM-spermidine 0.2 mM (0.058) as well as kinetin 0.5 mM-spermidine 0.5 mM (0.042). Good results were also obtained with spermidine 0.2 mM (0.063), spermine 0.2 mM (0.036) and GA_3 (0.5 mM: 0.032, 0.2 mM: 0.125). Putrescine 0.5 mM (0.013), spermidine 0.5 mM (0.012) as well as spermine 0.2 mM (0.005) gave exceptionally poor results, even when compared with control (0.021). Kinetins performed average in l:f score (0.026, 0.028). However, when perceived in larger perspective, these growth regulators were designated as beneficial: high limonoids (0.011, 0.012 units) and low fatty oils (0.43 units) resulted in good l:I (0.033, 0.039) and l:f (0.026, 0.028) ratios. Control samples were the reference mark for limonoids (0.010 units), fatty oils (0.49 units), l:I (0.028) as well as l:f (0.021).

According to Robins and Sweeney (1979), growth regulators form a metabolic link with limonoids (alkaloids). Potential polyamine precursors are efficiently incorporated into the alkaloids. This pathway is, infact, well characterized in several higher plant systems (Smith 1985). Synthesis of putrescine via arginine-agmatine pathway is in accordance with alkaloid metabolism. Putrescine is a dynamic branching point, being a substrate for both reversible spermidine formation and alkaloid synthesis (Hartmann *et al.* 1988). In contrast to the highly dynamic character of putrescine/spermidine, alkaloids accumulate as a virtually inert metabolic product. Therefore, the regulation of substrate flow from the dynamic polyamine pools was enhanced. Limonoid analysis indicate that spermidine might be an important control element, since the polyamine by itself or in combination was quite effective. The flow of metabolic pathway from arginine into the alkaloid-specific pathway seem to be sensitively controlled in the presence of putrescine.

According to the eco-physiological function of alkaloids, that is, as an insect repellent, there is only the need to maintain a suitable concentration of alkaloids within tissues to fulfill this function (Hartmann *et al.* 1988). A metabolic coupling of alkaloid synthesis to polyamine metabolism would offer a simple mechanism to adjust the required alkaloid level to growth rate. Active growth and cell division have been found to be correlated with increased rates of growth regulators (Sabherwal and Dhir 2009,10).

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