0971-4693/94 US $ 5.00
Allelopathy Journal 33 (2): 237-244 (2014) *International Allelopathy Foundation* 2014
Table: -, Figs : 2

Herbivore feeding stress on castor plants: Effects of different feeding modes on leaf alkaloids content

PATHIPATI USHA RANI

Biology and Biotechnology Division,
CSIR-Indian Institute of Chemical Technology, Taranaka, Hyderabad 500 007 (AP). India.
E. Mail: usharanipathipati@gmail.com , usharani65@yahoo.com

(Received in revised form: December 23, 2013)

ABSTRACT

Feeding by three pests, *Spodoptera litura, Ariadne merione* and *Liriomyza trifolli* changed the contents of total alkaloids and specific alkaloid ricinine in the leaves of castor (*Ricinus Communis* L.) plants. The mechanical damage of the plant also produced major changes in the plant's responses and in alkaloids content. The composition of total alkaloids content was affected due to feeding modes of different pests and mechanical damage. Quantitative determination of ricinine alkaloid by High Performance Liquid Chromatography (HPLC) showed that alkaloid ricinine was enhanced in plants by pests and mechanical damage.

Key word: Alkaloids, *Ariadne merione*, castor, feeding modes, *Liriomyza trifolli*, mechanical damage, ricinine, *Ricininus communis, Spodoptera litura*

INTRODUCTION

The importance of insect herbivory is increasingly recognized in ecological studies, hence, understanding the effects of herbivory on plant's defensive reactions is gaining importance in recent years. The development of modern analytical techniques contributed greatly to the identification of newly evolved plant's vast array of defence mechanisms for avoiding herbivore or pathogen damage. It is now evident that pest or pathogen damage leads to the qualitative or quantitative increase in the production of chemicals which take part in active plant defence (10). Chemical defenses are mostly associated with plant protection against biotic stress, providing advantages that lead to successful survival of plant species against herbivore insects. Among the plants produced chemical compounds in response to the herbivore attack, alkaloids attained an important status in recent years.

The herbivore induced leaf defensive compounds that can reduce subsequent damage are often referred to as anti herbivore compounds. Alkaloids are mostly associated with defense strategies against herbivore insects (3). Alkaloids are one among such groups of bioactive natural products which are nitrogen containing low molecular weight compounds. Alkaloids can be classified into many classes based on the nitrogen-containing ring system and their biosynthetic origin (11). Most research on plant alkaloid biosynthesis has focused on alkaloids such as benzylisoquinoline, monoterpenoid indole, tropane, purine, pyrrolizidine and quinolizidine types till recent (6). Pyridine nucleotides are involved in many other defense and signaling reactions (5). Pyridine alkaloids derived from pyridine nucleotides have also similar activities. One of the best studied pyridine alkaloid is nicotine, the first insecticide used to control the agricultural pests. It is extremely toxic to most herbivores (18). Ricinine, first toxic pyridine alkaloid isolated in 1864 (21), is major component in seeds and leaves of *Ricinus communis* (19) and is toxic to many pests. Several genera of Papaveraceae families produce alkaloids (benzophenanthridine alkaloid sanguinarine and protoberberine alkaloid berberine) which are insect deterrent and insecticidal.

Physical damage increases the production of nicotine in *Nicotiana tobacum* and cucurbitacin in leaves of *Cucurbita andreana* (20) as a part of their self defense. Pests such as leaf miners, sap-sucking insects, and other herbivores also higher chemical profile in plants on feeding (16). The Colorado potato beetle (*Leptinotarsa decemlineata*), is a serious pest of Potato and is not repelled by low concentrations of alkaloids such as solanine present in tubers, but plants of *Solanum chacoense* containing the acetylated glycoalkaloids (leptine) are highly resistant to these potato beetles (26). Seeds of *Lupinus albus* and *Lupinus mutabilis* contain up to 5% quinolizidine alkaloids in green parts of seeds. These seeds are bitter in taste and toxic to insects as well as to vertebrate, indicating the role of alkaloids in plant resistance to herbivores (25).

The castor [*Ricinus communis* (L.) (Euphorbiaceae)] is a major commercial crop in South India. Its oil has great medicinal value and is used in many pharmaceutical preparations. Castor has high medicinal role to maintain the disease free healthy life. Traditionally the plant is used as laxative, purgative, fertilizer and fungicide etc. whereas the plant possess beneficial effects such as anti-oxidant, antihistamic, antinociceptive, antiasthmatic, antiulcer, immunemodulatory, antidiabetic and hepatoprotective (14). Castor plant is attacked by several pests [*Ariadne merione* C. (Lepidoptera: Nymphalidae), *Spodoptera litura* F. (Lepidoptera: Noctuidae) and *Liriomyza trifolli* B. (Diptera: Agromyzidae)]. *A. merione* (common castor butterfly) feeds exclusively on castor plant through out the year and cause extensive defoliation. Tobacco cut worm, *S. litura* is a polyphagous pest, widely distributed throughout Asia and cause serious damage to the *R. communis* along with 112 other economically important crop plants (8, 17). *L. trifolii* is known as leaf miner which destroy the leaf mesophyll and it has a wide host range including castor bean.

Castor plant defence mechanisms were studied for changes in the alkaloid contents due to the feeding by 3 pests of castor plant having different modes of feeding as well as mechanical damage. The variation in total alkaloid content, the quantity of alkaloid ricinine in *R. communis* plants was determined using analytical techniques, after feeding damage caused by different pests and also by mechanical damage and were compared to normal healthy and pest uninfested castor plants. Our previous studies documented the quantitative and qualitative increase of several enzymes (9), secondary metabolites like phenols and phenolic acids (22, 23) in pest fed *R. communis* plants. However, the detailed information on changes in total alkaloid content in castor plants due to herbivore feeding and mechanical damage has not yet been reported. Hence, castor plant defense responses to the feeding of three pests and mechanical damage were studied by monitoring the alkaloid content of *R. communis*.

MATERIALS AND METHODS

Insect~~s~~ Rearing: Common castor butterfly [*Ariadne merione* C. (Lepidoptera: Nymphalidae)] and the tobacco cutworm [*Spodoptera litura* F. (Lepidoptera: Noctudae)] were obtained in their egg stages from the fields of Acharya N.G. Ranga Agricultural University, Hyderabad and cultures were maintained in our laboratory [temperature 28 ± 2°C, 65 ± 5 % RH and a photoperiod of 16:8 h L: D] on fresh castor (*Ricinus communis* L.) leaves . Healthy third instar larvae of *A. merione* and *S. litura* were used for the experiments. The cultures of leaf miner *Liriomyza trifolli* B. (Diptera: Agromyzidae) were maintained in laboratory and the infested leaves were collected for the experiments.

Plant Growth: Plants of Castor (*Ricinus communis* L.) variety 'Aruna' were used to rear the pest insects and for experiments due to its susceptibility to all kinds of pest attack. Plants were grown in laboratory on large scale to use the foliage for insect culture. Seeds were also sown in plastic pots (30 x 40 cm dia) kept in green house [12h: 12 h light dark, 25±2ºC and relative humidity 65±5 % RH] for use in experiments. Sixty d old castor plants grown in pots were taken in laboratory for pest feeding assays.

Chemicals: Bismuth nitrate pentahydrate, thiourea, disodium sulfide and all other standards used were purchased from Sigma Aldrich (purity ~ 99%). HPLC grade solvents and reagents were used for alkaloid estimations (Merck chemicals, Darmstadt, Germany). The water used was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Pest feeding: For obtaining pest damaged castor leaves, *R. communis* plants of 60 day old were infested individually with pre starved healthy 3rd instar larvae of *A. merione* and *S. litura.* These insects were released separately on terminal leaf of each plant and confined at the point of release by enclosing it in a muslin bag. They were allowed to feed on the leaves for 6 h and the remaining leaf portions after feeding, which usually consisted of approximately 40-50% of the total-leaf. At the end of 6 h feeding period the insects were removed, and then the pest damaged leaves were collected after 24 h and used for alkaloids assay. Similarly the *L. trifolli* infested leaves were collected from the fields for their phytochemical analysis. In all experiments, fresh leaves of similar age from healthy castor plants without any pest infestation were used as controls for comparison.

Total alkaloids content: The total alkaloids content from the pest infested leaves was analysed and compared to normal healthy leaves. The alkaloid extractions were done using the method described by Lee and Waller (12). Briefly, 5 g tissue was frozen with liquid N_2, homogenized in 80 % MeOH until the residue was free of soluble pigments. The organic solvent was removed from the pooled extracts by evaporation under reduced pressure. The left over aqueous solution was subjected to extraction by light petroleum to remove lipid and pigments. Remaining aqueous phase, which contained alkaloids was evaporated to dryness. The dry residue from the aqueous portion of extract was separated using boiling MeOH, the volume was used for the total alkaloids content and quantitative analysis of ricinine in the sample.

The total alkaloids content (TAC) in methanolic leaf extracts was estimated. For this, 5 mL amount extract (pH 2.5) was mixed with 2 mL of Dragendorff's reagent and centrifuged at 9223 x g for 25 min, the residue obtained was treated with 2 mL disodium sulphide solution. The precipitate thus obtained was dissolved in 2 mL of HNO_3 (conc), diluted distilled water. One mL of the extract was added to 5 mL thiourea solution and the absorbance was measured at 310 nm (TECHCOMP vis 7200 visible spectrophotometer).

Isolation of ricinine: Pest infested plant materials of 50 g was weighed and freeze dried with liquid nitrogen overnight, extracted in 400 mL of methanol. The extract was filtered and filtrate was evaporated to dryness by rotavapor. The dried material (2.54 g) was collected and subjected to fractionation by column chromatography over silica gel (200g-acme's 100-200 mesh) and eluted with hexane and methanol solvents of increasing polarity. Ethyl acetate fraction (0.375 g) was further subjected to column chromatography with 90% chloroform in ethyl acetate to yield ricinine fraction (66 mg) and the separation of the compound was monitored by TLC with mobile phase [chloroform : methanol : 7% acetic acid 5:1:1]. TLC plates are visualized by spraying with dragendroff's reagent, observed for color development and identified the compound in orange colour against yellow background with Rf values 0.75.

HPLC Analysis: The amount of alkaloid ricinine was quantitatively determined using HPLC as per method of Liu *et al.* (13). The 50 µL of solution injected into a reversed phase C_{18} column with a simple isocratic elution of 70% solvent A (10% methanol in 90% water), and 30% solvent B (100% methanol) used as mobile phase (flow rate of 0.8 mL/ min) for 9 min. An Agilent Technologies HPLC Model 1100 equipped with a diode array detector was used for the quantitative analysis of the ricinine and was determined at its absorbance of 310 nm.

Statistical analysis: Total alkaloid content and quantification of ricinine experiments consisted of 4 replications and all the experiments were performed in similar conditions and were repeated thrice. The data of different treatments were subjected to one way ANOVA and Tukey's HSD using AnalystSoft, Biostat analysis program (Biostat, 2009) to determine the main differences in treatments.

RESULTS AND DISCUSSION

Approximately 20% of higher plants produce alkaloids and these compounds often exhibit antifeedant or toxicity to herbivores. This study relates to the changes in TAC in response to different pest feeding modes and mechanical damage. Induced responses observed were similar in both pest and mechanical damaged *R. communis* plants were similar. Significant changes in TAC and ricinine occurred in both pest infested and mechanically damaged plants (Fig. 1). There was almost 35% increase in the alkaloid content in *R. communis* plants infested with different pests. The total alkaloid content in normal uninfested castor plants was 4.03 mg/G FW and infestation with *L. trifolli, S. litura and A. merione* resulted in elevated levels of TAC by 1.3, 1.33 and 1.38 times respectively in castor plants in comparison to control plants. Mechanical damage also induced an increase in total alkaloid concentration to 4.96 mg/G FW. This increase was lower than pest infested TACs suggesting that plants not only respond to level of damage but also depends on the pest which causes the damage. Among all the three pests studied, *A. merione* infestation increased alkaloids content to 5.6 mg/G FW compared to normal plants. *L. trifolli* and *S. litura* showed TAC increases to 5.4 and 5.3 mg/G FW, respectively (Fig. 1). In several plants, the alkaloid levels increased after herbivory damage (2). *R. communis* plants exhibited significant differences of TAC in response to different pests. Pest infestation had higher total alkaloid content induction than mechanical damage. Responses to pest damage are in partial agreement with those reported previously in phenolics estimation bioassays with different pests [*Achaea janata, Spodoptera litura, Dichocrocis punctiferalis* and *Empoasca flavescens* (9)]. Previous work suggested that the levels of glutamine, ammonia, urea, total carbohydrate, proteins and total phenol increased upon feeding by leaf eating insects than sucking pests or mechanically damaged plants.

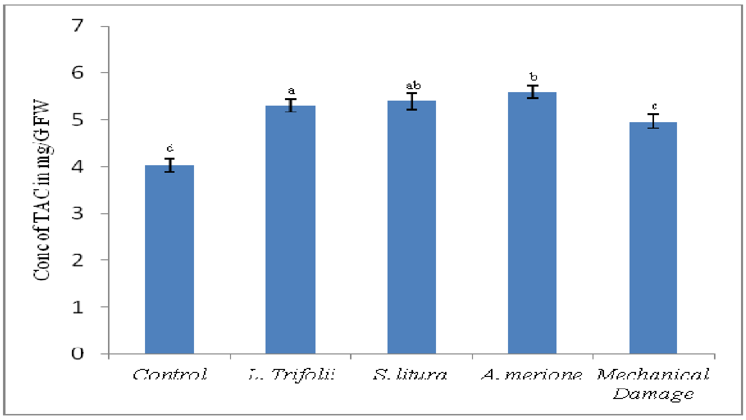

Figure 1. Effects of feeding by three insect pests on the Total alkaloid content in castor leaf extract. Bars with the same letters are not significantly different at P = 0.05 (Tukey HSD test)

HPLC analysis

There were qualitative and quantitative differences in ricinine content between control and pest/mechanical damaged plants of *R. communis*. The control leaf samples had the sharp ricinine peak (retention time=3.8 min) and its concentration was 0.03 mg/G FW. A significant difference in quantities of ricinine was observed due to herbivory by all three castor pests tested as well as in mechanical damage (Fig. 2). The level of ricinine in castor was also enhanced in response to green peach aphid *Myzus persicae* (Sulzer) feeding (15). Ricinine abundance was higher in the pest damaged leaves compared with mechanical damaged and normal/control plants.

Among the castor pests that fed on the host plants, *A. merione* feeding induced the highest production of ricinine. An increase of 0.281 mg/G FW ricinine was observed in case of *A. merione* damaged castor leaf extracts while an increase of 0.248 and 0.148 mg/G FW was observed in case of *L. trifolli* and *S. litura*, respectively (Fig. 2). Similar enhancement of alkaloid content was reported in the leaves of *Lobelia cardinalis*

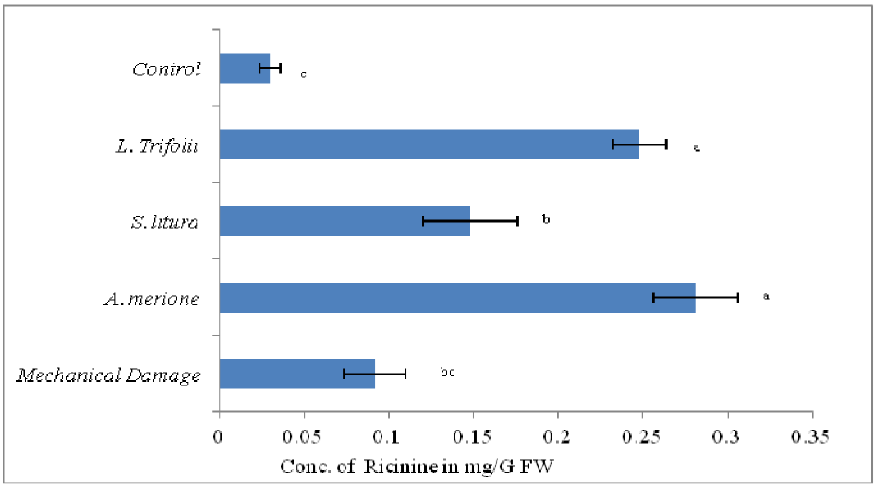

Figure 2. Effects of feeding by three insect pests on the ricinine content in castor leaves. Means followed by same letters are not significantly different at P=0.05 (Tukey HSD test)

(Campanulaceae) due to feeding by plusiine caterpillar, *Enigmogramma basigera* (Lepidoptera: Noctuidae) and by leaf miners, sap-sucking insects (16). The density of leaf damage due to *A. merione* feeding is higher when compared to the damage-caused by *S. litura* feeding. Though both the pests are chewing pests, these responses may be due to the effect of increased alkaloid content as chemical defenses which inhibit further feeding of *S. litura.*

Leaf miners feed on palisade mesophyll of leaves that have more proteins for their growth and development, and as alkaloids are transported through mesophyll cells (1), they could have evolved new mechanisms to tolerate the higher amounts of alkaloids compared to other insects. Similar results were reported by Filho and Mazzafera (7) where they observed that leaf coffee miner *Perileucoptera coffeella* can tolerate the presence of the alkaloid caffeine in order to consume the high protein quality containing mesophyll tissues where alkaloids are in high concentrations. This quantitative enhancement in ricinine content has also occurred due to mechanical damage and an increase of 0.092 mg/G FW was observed. The elevated level of alkaloid cucurbitacin in the leaves of *Cucurbita andreana* was observed when subjected to mechanical damage (20). It is also reported that the mechanical wounding in *Catharanthus roseus* had a profound effect on alkaloid metabolism and increase in ajmalicine accumulation was observed (24).

Feeding by pests caused greater increase in ricinine levels than with mechanical damage. Chludil *et al.* (4) suggested that while mechanical damage triggers the same response in alkaloid production, there were no differences in total alkaloid content on level of leaf damage in *Lupinus sps*. These variations may be attributed to both the duration of pest contact with plant as well as its quantitative damage and this could have played a vital role in the enhancement of alkaloid content in *R. communis* plants. Previous work by Jyothsna *et al.* (9) on *Achaea janata, Spodoptera litura, Dichocrocis punctiferalis* and *Empoasca flavescens* showed increase in quantities of primary and secondary metabolites [amino acids, glutamine, ammonia, urea, total carbohydrates, proteins and total phenols], which varied with different feeding modes. The increase in concentrations due to feeding of leaf eating insects was higher than for sucking pests or mechanically damaged plants. Increase in alkaloid and other metabolites concentration after damage would more effectively deter the herbivores than at normal levels, because of change in the palatability, as the herbivores would perceive the change and stop consuming damaged tissues. Due to change in food quality, herbivores move to more palatable plants or plant parts.

CONCLUSIONS

The present results contributed in exploring the importance of alkaloids in plant defence mechanism, used to limit the herbivore damage. The relatively less elevation of total alkaloid content in mechanical damaged plants suggest that the plants perceive the physical damages as an insect attack and protect the leaf from further infestation of generalist insect herbivores. Thus feeding of herbivorous insects induces antibiosis based on accumulation of the alkaloids in castor plants. An elevated level of alkaloid content was evident in pest and mechanical damaged *R. communis* plants than uninfested plants. In particular, Individual alkaloid concentration showed that alkaloid ricinine levels increased in response to feeding by three insect pests and by mechanical damage. Feeding by leaf eating insect pests showed several significant challenges in the concentration of total alkaloid content as well as ricinine in the leaves of *R. communis*. The leaf alkaloid levels were modified quantitatively due to pest feeding and further these enhanced alkaloids varied differently among the three tested insects-having different modes of feeding as well as in case of mechanical damage.

REFERENCES

1. Barbehenn, R.V., Reese, J.C. and Hagen, K.S. (1998). The food of insects, pp. 83-121, In: *Ecological Entomology* (Eds., C. B. Huffaker and A. P. Gutierrez). 2nd ed. John Wiley & Sons, New York.
2. Bashir-Khan, M. and Harborne, J.B. (1990). lnduced alkaloid defence in *Atropa aeuminata* in response to mechanical and herbivore leaf damage. *Chemoecology* **1:** 77-80.
3. Beninger, C.W., Abou-Zaid, M.M., Kistner, A.L.E., Hallett, R.H., Iqbal, M.J., Grodzinski, B. and Hall, J.C. (2004). A flavanone and two phenolic acids from *Crysanthemum morifolium* with phytotoxic and insect growth regulating activity. *Journal of Chemical Ecology* **30:** 589-606.
4. Chludil, H.D., Vilarino, M.P., Franco, M.L. and Leicach, S.R. (2009). Changes in *Lupinus albus* and *Lupinus angustifolius* alkaloids profiles as response to mechanical damage. *Journal of Agricultural and Food Chemistry* **57:** 6107-6113.
5. Crawford, N.M. and Guo, F.Q. (2005). New insights into nitric oxide metabolism and regulatory functions. *Trends Plant Science* **10**: 195-200.
6. Facchini, P.J. (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annual Review of Plant Physiology and Plant Molecular Biology* **52:** 29-66.
7. Filho, O.G. and Mazzafera, P. (2000) Caffeine does not protect coffee against the leaf miner *Perileucoptera coffeella. Journal of Chemical Ecology* **26**:1447-1464.
8. Hadapad, A., Chaudhari, C.S., Kulye M., Chaudele, A.G. and Salunkhe, G.N. (2001) Studies on chitin synthesis inhibitors against gram pod borer, *Helicoverpa armigera* (Hub.). *Journal of Nature Conservation* **13**: 137-140
9. Jyothsna, Y., Kapil, M. and Usha Rani, P. (2009) Effects of herbivore feeding on biochemical and nutrient profile of castor bean, *Ricinus communis* L. plants. *Allelopathy Journal* **24**: 131-142.

10. Karban, R. and Baldwin, I.T. (1997). *Induced Responses to Herbivory*. The University of Chicago Press, Chicago, IL.
11. Khadem, S. and Marles, R.J. (2012) Chromone and flavonoid alkaloids: Occurrence and bioactivity. *Molecules* **17:** 191-206.
12. Lee, H.J. and Waller, G.R. (1972). Biosynthesis of pyridine alkaloids from *Tripterygium wilfardii*. *Phytochemistry* **11**: 2233-2240.
13. Liu, X., Zhang, Y., Li, H. and Li, D. (2005). Determination of ricinine plasma concentration and its preliminary pharmacokinetic study in rats. *Asian Journal of Drug Metabolism and Pharmacokinetics* **5:**133-137
14. Mir, M.I. (2013). Phyto and pharmaceutical value of *Ricinus communis* L.: A review. *International Journal of Pharmaceutical Research and Development.* **5:** 14-23.
15. Olaifa, J.I., Matsumura, F., Zeevaart, J.A.D., Mullin, C.A. and Charalambous, P. (1991). Lethal amounts of ricinine in green peach aphids (*Myzus persicae*) (suzler) fed on castor bean plants. *Plant Science.* **73:** 253-256.
16. Oppel, C.B., Dussourd, D.E. and Garimella, U. (2009). Visualizing a plant defense and insect counter ploy: alkaloid distribution in *Lobelia* leaves trenched by a plusiine caterpillar. *Journal of Chemical Ecology* **35**: 625-634.
17. Paulraj, M.G. (2001). *Integration of Intercrops and Plant Product on Chosen Groundnut Pests Management.* Ph.D thesis, St. Joseph's College (Autonomous) Bharathidasan University. Trichy, Tamil Nadu, India.
18. Schmeltz, I. (1971). Nicotine and other tobacco alkaloids. In: *Naturally Occurring Insecticides.* (Eds., M. Jacobson and D.G. Crosby). New York: Marcel Dekker. pp. 99-136.
19. Skursky, L. and Waller, G.R. (1972). In Abh. Dtsch. Akad. Wiss. Kl., Chem. Geol. Biol., Berlin; 4. Internationales Symposium Biochemie und Physiologie der Alkaloide, June, 1969, Halle (Saale) Germany, pp. 181-186.
20. Tallamy, D.W. and McCloud, E.S. (1991). Squash beetles, cucumber beetles, and inducible cucurbit responses. pp. 155-181. In: *Phytochemical Induction by Herbivores*, (Eds., D.W. Tallamy and M.J. Raupp). John Wiley, New York.
21. Tuson, R.V. (1864). Note on an alkaloid contained in the seeds of the *Ricinus communis*, or castor-oil plant. *Journal of Chemical Society* **17:**195-197.
22. Usha Rani, P. and Pratyusha, S. (2013) Defensive role of *Gossypium hirusutum L.* antioxidative enzymes and phenolic acids in response to *Spodoptera litura F.* feeding. *Journal of Asia Pacific Entomology* **16**: 131-136.
23. Usha Rani, P. and Ravibabu M.V. (2011). Allelochemicals in castor (*Ricinus communis*) plants and their impact on pest larval feeding as anti-herbivore defensive. *Allelopathy Journal* **27**: 263-276.
24. Vazquez-Flota, F., Carrillo-Pech, M., Minero-García, Y. and De Lourdes Miranda-Ham, M. (2004). Alkaloid metabolism in wounded *Catharanthus roseus* seedlings. *Plant Physiology and Biochemistry* **42:** 623-628.
25. Wink, M. (1987). Chemical ecology of quinolizidine alkaloids. In: *Allelochemicals: Role in Agriculture, Forestry and Ecology* (Ed., G.R. Waller). American Chemical Society Symposium Series No. **330**: 524-533.
26. Wink, M. (1988). Plant Breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theoretical and Applied Genetics* **75**: 225-233.