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Potential Insecticidal Effects of *Azadirachta indica* and *Nicotina tabacum* against the Gall Wasp, *Leptocybe invasa* (Hymenoptera, Eulophidae: Tetrastichinae) on *Eucalyptus grandis* Seedlings

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ABSTRACT

Easier access to botanicals and the return to traditional means of pest control have cast a new spell on botanicals. Botanicals are cheap, efficient and not prone to induce resistance in the pest. Among the studied botanicals used as pesticides worldwide, the neem tree and the tobacco plant have proved to be the richest in active compounds and are some of the potent sources of natural biocides. Bioassays were carried out on the gall wasp *Leptocybe invasa* on *Eucalyptus grandis* using neem, tobacco and neem/tobacco mixture each at concentrations of 5, 10 and 15g/l and observations on oviposition, gall development and adult emergence were made at two week intervals for a period of 12 weeks. Results showed that tobacco had the highest insecticidal effects in reducing oviposition rate, whilst the mixture of neem/tobacco reduced gall formation. Neem was potent at reducing adult emergence and for all extracts, an increase in concentration led to a decrease in oviposition, gall formation and adult emergence. Results from this study point to the feasibility of using these extracts in the control of *L. invasa* on *Eucalyptus* from the devastation of this pest.

Introduction

The blue gum chalcid (*Leptocybeinvasa*) tops the list of the most severe invasive *Eucalyptus* species pests that are affecting plantation forestry on an international level. *L. invasa* is native to Australia where it is a major pest of *Eucalyptus* trees. It became a major pest from the year 2000, spreading from the Middle East and Mediterranean region, and ever since, it has spread to all continents where there are *Eucalyptus* trees in a period of 10 years.

Leptocybe invasa falls under the super family Chalcidoidea. Its common name is the blue gum chalcid and it comprises only of a single species, *L. invasa*. The wasp has a brown body that has a slight green to blue iridehiscence and attains a length of just over a millimeter at maturity (Hurley, 2015)

Eucalyptuses belong to the Myrtaceae family,which is a broad family that comprises a genus of shrubs and angiosperm trees, the *Eucalyptus* genus. The flora of Australia is dominated by this family's members and more than 700 species of *Eucalyptus* are native to Australia. At maturity *Eucalyptus* can take either of two forms; a small shrub or a very huge tree.

Young *Eucalyptus* trees, especially the seedlings and those not more than a year are usually the ones that are highly affected by *L. invasa*. The adult female wasp, whose length is not more than 1.2mm lays her eggs in the midribs, petioles and stems of juvenile trees as well as in recently produced coppice growth. This forms bump-shaped galls and leads to stunting of the tree during growth.

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A week or two after egg-laying, cork tissue begins to show at the point of insertion and the galls form inside the plant tissue surrounding the eggs. Gall development commences as soon as the eggs hatch and this is due to the maggot-like larvae inside the gall that will be feeding and growing until it grows to a required size. When maximum size of growth is reached, pupation of fully developed larvae occurs within the gall and adults emerge through holes they chew (Nyeko, 2005).

The galls form principally on young shoots, the midrib and on new leaves. However, due to heavy levels of infestations, tissues can be the target where egg laying occurs and in cases of heavy infestations, laying of eggs can cause galls to develop in tissues and the size varies. Sap flows out of ovipostion holes leading to formation of galls. Formation of galls on the terminal shoots of the leaves of *Eucalyptus* causes these shoots to dry out due to the deformation that will have occurred. When galling has reached its extremes, apical dominance is lost and retarded and stunted growth takes place in trees. Occurrences are most likely in nursery seedlings and plantations of about 2 years and below.

The insect has been successful in its spread on a worldwide level due to its biology. The female reproduces asexually without mating, a method known as thelytokous reproduction, and living for an estimated 7 days (Doganlar and Hassan, 2010). Only female offspring are produced by this type of reproduction. In South Africa, it was discovered that only 72 days are taken for the insect to complete its life cycle from egg to adult. *L. invasa* can tolerate a vast range of climatic conditions enabling it to survive in new environments and this has also aided in its spread from Australia to other parts of the world (Mendel *et al.,* 2004).

Chemical control has been one of the prominent methods of insect management over the years. Application of broad spectrum systematic insecticides may be effective in protecting nursery seedlings and plantation establishment; however, its effectiveness is not feasible over a long term period of control. Biological control is the best mode of control which has been judged effective (Makaka, 2008). Four species of natural enemies have been sent from Australia to Israel to control the insect. The best chance of control over a lengthy period is offered by these (Kelly *et al.,* 2012).

The use of botanicals is another way which can be considered effective in nurseries to reduce the effect of the blue gum chalcid on nursery seedlings. Two such botanicals which have been used extensively on other pests are neem and tobacco.

Azadirachta indica, a tree in the mahogany family, Meliaceae is also known as neem, nimtree or Indian Lilac. The genus *Azadirachta* has only two species, the neem tree being one of them and is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka. Characteristically, it has been noted to grow in tropical and semi-tropical regions and islands in the southern parts of Iran. Neem fruits and seeds are the source of neem oil (Kluwer, 2009).

Neem is one of the key ingredients in non-pesticidal management (NPM), as it provides a natural substitute to synthetic pesticides. A powder is made by grinding the seeds and soaking this in water will form a solution which will be sprayed onto a crop. Repeated application is necessary for the botanical insecticide to be effective and this is done at minimum every ten days. Insects are not killed directly as neem acts as an anti-feedant, repellent, egg-laying preventive and protects the crop from mutilation. The insects are starved to death which comes within a few days. Insects are also suppressed from hatching to larvae.

The curing of tobacco plant leaves gives a by-product which is commercially known as tobacco. Tobacco is a plant indigenous in North and South America, South West Africa, Australia and the South Pacific and is a member of the Solanaceae (nightshade) family under the genus *Nicotiana.* Nicotine is a potent neurotoxin to insects and many plants contain it. However, the concentration of nicotine in tobacco is higher than those in other plants. In addition, tobacco does not contain tropane alkaloids that are harmful to animals and human beings as do other members of the Solanaceae.

High infestation levels on Eucalyptus trees by *L. invasa* are decreasing the yields of these trees. Formation of galls on shoots and midribs of leaves blocks the translocation process and therefore trees do not grow to expected height due to nutrient deficiencies and in some cases this can ultimately lead to death of the plant.

The use of botanicals as pesticides is environmentally and socially acceptable. Synthetic insecticides can cause a problem as they can kill non-target organisms in the vicinity like birds feeding on the plants, as they are not specific.

Although biological control is effective on a large scale and plans to import *L. invasa* parasitoid *Selitrichodes neseri* from South Africa are underway, use of natural biochemical extracts in the nursery can be an effective way of controlling the pest and reducing its effects on Eucalyptus seedlings in nurseries (Mwangi and Mwangi, 2013).

The demand for wood and timber in Zimbabwe continues to grow. Eucalyptus plantations are a major source of timber, firewood and honey foraging. More importantly and of particular concern of late is the continued use of firewood for tobacco curing by newly resettled farmers. Eucalyptus trees are considered to be ideal to address the demand for wood because they grow fast and are adaptable to a wide range of sites. Commercial companies such as British American tobacco (BAT) also encourage the establishment of Eucalyptus woodlots as an alternative supply of timber and wood. *Leptocybe invasa* poses a threat to nursery seedlings and the establishment of such woodlots.

The main objective of the study was thus to determine the potential insecticidal effects of neem and tobacco extracts against *L. invasa* on *E. grandis* seedlings. Specifically, the study aimed at investigating the insecticidal effects of neem and tobacco on reducing oviposition by *L. invasa* on *E. grandis* seedlings and to determine the insecticidal effects of neem and tobacco against gall formation on *E. grandis* seedlings. The study also sought to determine the insecticidal effects of neem and tobacco extracts on *L. invasa* adult emergence.

Methodology

Area of study

The investigation was carried out at Forestry Commission which is in Highlands, Harare from mid-May to mid-September 2015.

Growing of host

A total of 150 seedlings of the species *Eucalyptus grandis* were sown into small pots in the nursery. The seedlings were watered to saturate the soil and thereafter, watered twice a day for 6 weeks. Growth was monitored in the 6 week period until a height of about 15cm had been reached.

Collection and culturing of pest

Branches and twigs which were highly infested with *L. invasa* galls were collected from *Eucalyptus* hybrids plantations around the Forestry commission site in Harare. These were pruned to remove leaves and shoots. The remaining stumps of highly infested *L. invasa* galls were taken to the laboratory where they were placed into black emergence plastic chambers with a height of 58,6cm and a diameter of 146cm.The emerging insects were collected through a pipe using a jar. After 2 days, the jar was removed and placed in the refrigerator for 5 minutes to inactivate the pest. After this the pests, *L. invasa*, in the jar were counted. A minimum number of 120 insects were required.

Preparation of Extracts

Concentrations of 5g/L, 10g/L and 15g/L were made by adding 5g, 10g and 15g of ground neem seeds to a liter of distilled water. Similar concentrations of tobacco extract were made by adding 5g, 10g and 15g of ground tobacco leaves into 1L volumes of water. Mixtures of neem and tobacco extracts at concentrations of 5g/L, 10g/L and 15g/L were also prepared.

Application of Plant Extracts

The 150 potted seedlings were arranged in rows of 5 in a rectangular seed bed. Each of the 3 neem extract concentrations were applied to 5 seedlings with each concentration restricted to a row. Application involved pouring and spraying 30ml of each concentration to a single potted plant. This procedure was repeated for tobacco extract concentrations as well as the tobacco/neem mixture concentrations. Each treatment was replicated three times and treatments were randomized. A control, comprised of distilled water, without any extract was also included.

Introduction of Pest

The jar containing the 120 insects was refrigerated to immobilize the insects and equal numbers put into each of 3 jars. All the seedlings in the seed bed were covered by a cloth veil and firmly secured in place along the edges using bricks. The veil was partially opened along the edges at one meter intervals and each of the 3 jars was simultaneously opened to release the pest and quickly covered again. The setup was left overnight and the jars and the veil were removed the next day. An assessment of oviposition and gall formation was carried out after 2 weeks. This was followed by repeated applications of extracts onto the seedlings, pest introductions and assessments of seedlings at 2 weeks intervals for 12 weeks.

Assessments

Assessment of oviposition

Assessments were done after every 2 weeks after treating seedlings with extracts and re-inoculation of seedlings with the pest. Assessments involved looking for early signs of peeling off, of the upper epidermis of seedlings stems (an indication of pest invasion). This was done until development of galls commenced and time taken for oviposition to occur was noted.

Assessment of gall formation and development

The number of galls formed at each 2 week interval and the cumulative number of galls for each plant for the 12 weeks were recorded. Average galls formed were also calculated for each treatment.

Assessment of adult emergence

For each treatment, signs of adult emergence were also assessed by examining the galls on each plant. The number of emergence holes after the 12 weeks were counted for each plant and the average emergence holes per treatment was calculated.

Data analysis

Results were analyzed using two way ANOVA using SPSS version 22, with type of extract and extract concentration as factors and gall development and adult emergence as the response variables.

Results

The effect of botanicals on oviposition

The tobacco extract was the most potent in reducing oviposition as indicated by the low levels of peeling. All tobacco extract concentrations fared better as compared to the other extracts. This was followed by the tobacco/neem mixture concentrations and the neem extract was the least potent as indicated by the relatively high levels of peeling. However, all extracts at all concentrations did better than the control.

The effect of the botanicals on gall formation and development

At the second and fourth weeks of assessment, no galls were formed in all treatments and at all concentrations. From the sixth week of assessment until the12th week, there was a general increase in the number of galls for all extracts across all concentrations. However, the number of galls developing reduced with concentration for all treatments (Figure 1).

The tobacco/neem mixture was most effective at reducing gall development at all concentrations (Figure 1); this was followed by neem extract, while the tobacco extract proved to be the least potent. All extracts showed limited effectiveness at the lowest concentration of 5g/L. The control showed the highest increase in gall development with 43galls by the 12th week. Anova results showed that type of extract has an effect on gall formation ($P<0.05$), however, there was no significant difference between the three concentrations except for neem at 5g/L and 15g/L (Figure 1).

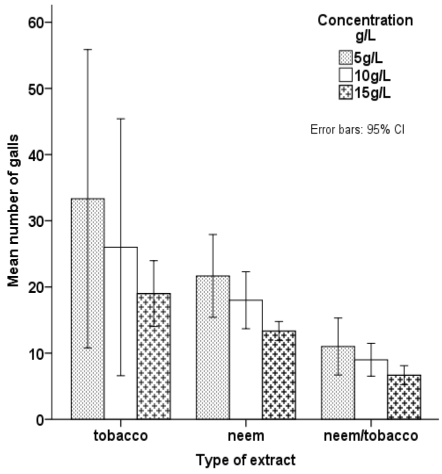

Figure 1. Mean number of galls formed during the 12 week period for the three types of extracts, at the different concentrations.

Effect of tobacco extract on gall formation and development

Figure 2a shows gall development when tobacco was used at three concentrations against the control. Results showed an increase in gall formation with time and a reduction in gall formation as concentration increased. An average of 33 and 20 galls was reached within 12 weeks at 5g/L and 15g/L of tobacco respectively.

Effect of neem extract on gall formation and development

Figure 2b shows mean number of galls for all three concentrations of the neem extract. For all concentrations, the numbers of galls were less than 20 at the 12th week of assessment. There was a general increase in gall development from the second week until the twelfth week at all concentrations (Figure 2b). The control had an average of 40 galls after 12 weeks. The neem extract therefore showed high pesticidal effects on the formation of galls and their development compared to the control and the other two botanicals.

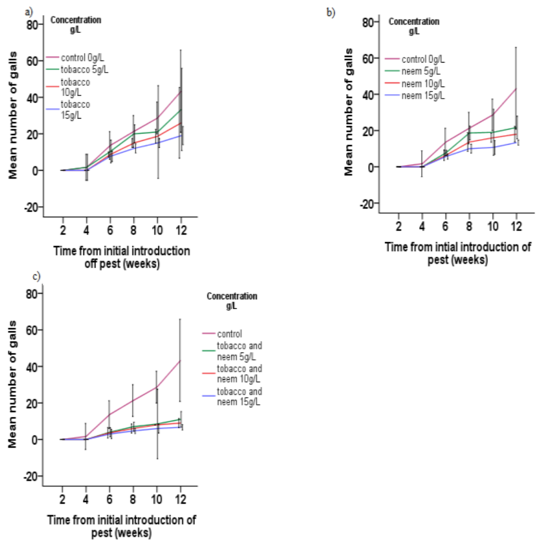

Figure 2. Number of galls formed after initial introduction of pest and initial treatment with (a) tobacco, (b) neem and (c) neem/tobacco mixture at different concentrations.

Effect of neem/tobacco mixture on gall formation and development

Gall formation and development was reduced greatly by the neem-tobacco extract at the three different concentrations. Gall formation varied amongst all concentrations with 15g/L recording a lower number of galls than the other concentrations as time progressed. Therefore, an increase in concentration showed a reduction in gall development compared to the control. After 12 weeks, less than 10 galls had been formed on average for all three concentrations with the control having more than 40 galls (Figure 2c).

The effect of the botanicals on emergence of adults

The insecticidal effect of the extracts was also tested for on the emergence of adults of the pest *L. invasa*. During the first 6 weeks, assessments showed that no adults emerged from the galls. Emergence holes were first observed in the eighth week. The mean numbers of emergence holes in the three extracts are shown in Figure 3. There was a general increase in adult emergence with time at all concentrations for all three plant extracts including the control. On the other hand, there was a general decrease in adult emergence with increasing concentration for all extracts (Figure 3).

The three extracts showed some variations in number of adults that emerged at the different concentrations. Neem extract was most potent in reducing adult emergence holes (an average of 6 emergence holes at concentration 15g/L) at 12 weeks whilst tobacco was the least effective (an average of 17 emergence holes at a concentration of 15g/L). The tobacco/neem mixture extract came second in effectiveness with an average of 12 adults emerging at 15g/L (Figure 3). The control showed the highest number of emergence holes reaching up to 49 emergence holes by the twelfth week in one replicate. Anova results supported the point that emergence of adults was significantly affected by concentration of extract ($P<0.05$).

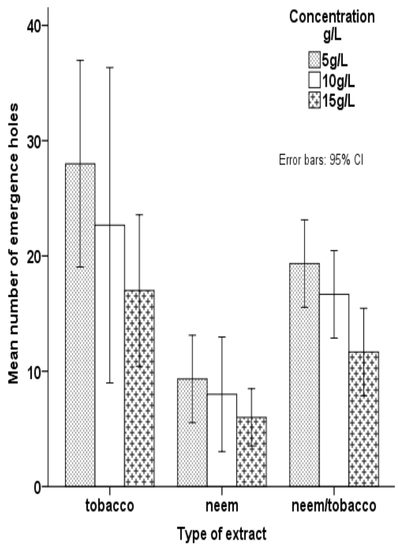

Figure 3. Mean number of emergence holes formed during the 12 week period for the three types of extracts, each at three different concentrations.

Effects of tobacco extract on adult emergence

Tobacco showed the least effectiveness in inhibiting adult emergence. The highest concentration of the tobacco extract (15g/L) had an average of 17 adults emerging by the 12th week whilst the 10g/L concentration had an average of 23 adults emerging by the twelfth week (Figure 4a). The lowest concentration of the tobacco extract (5g/L) had seedlings with the highest number of emergence holes.

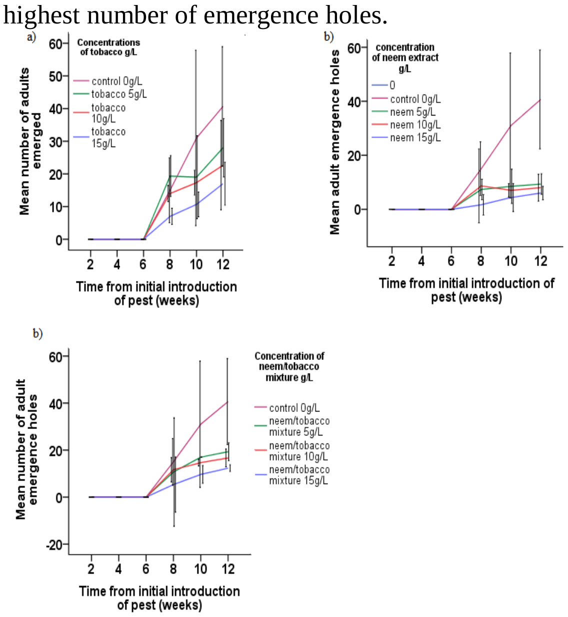

Figure 4. Number of adult emergence holes after initial introduction of pest and initial treatment with (a) neem, (b) tobacco and (c) neem/tobacco mixture at different concentrations

Effects of neem extract on adult emergence

Amongst all three extracts, neem seemed to exhibit the highest insecticidal effects. The seedlings treated by neem had less emergence holes meaning, less adults emerged from these. The higher the concentration, the fewer the number of adults that emerged. Neem at 5g/L, 10g/L and 15g/L had 9, 8 and 6 emergence holes respectively by the 12th week (Figure 4b) and these were less than the other two treatments for tobacco and tobacco and neem (Figure 4).

Effects of neem/tobacco mixture on adult emergence

The neem-tobacco extract was shown to be less effective as an insecticide than neem but better than tobacco which showed the least effect (Figure 4c). This trend was also observed for all the 3 concentrations for these 2 treatments. Figure 4c shows averages of 19, 17 and 12 adult emergence holes for 5g/L, 10g/L and 15g/L of neem/tobacco mixture respectively after 12 weeks. Higher concentrations therefore showed a decrease in emerging adult numbers.

Discussion

Oviposition

Oviposition is the laying of eggs by an insect. In the case of *L. invasa*, evidence of oviposition was seen by the slight peeling off of the upper epidermis which was witnessed upon assessment after the first four weeks after the initial introduction of the pest. The least peeling off of the epidermis was on the seedlings treated by tobacco at all 3 concentrations with those treated with 15g/L showing the least peeling off. This could be attributed to the contact insecticidal effect of the nicotine in the tobacco. Of the 3 extracts, neem showed the least potency and this could be because azadirachtin has systemic insecticidal effects. The mixture of tobacco and neem had both the contact and systemic insecticidal effects respectively and therefore the nicotine again impeded oviposition but was diluted by neem and worked less effectively compared to the tobacco extract. Galling in *Eucalyptus* species is evidence enough to show that egg laying by the pest *L. invasa* occurred and the eggs hatched into larvae.

Gall formation and development.

Many compounds such as limonoids, azadirone, azadirachtin, and flavonoids, having therapeutic potential, have been isolated from various parts of neem tree and have been evaluated for their pharmacological actions (Mahapatra *et al*, 2012). Neem is grown throughout the world mainly for its use as a natural pesticide. In addition to being an insecticide, it has been used as a fungicide, nematicide and bactericide. Commercial products made with neem include Bioneem, Margoan-O, Biotrol and Nimex. The active ingredient in neem mimics an insect hormone and repels insects, as well as inhibiting their digestion, metamorphosis and reproduction. It has been used effectively on over 100 leaf-eating insects. As shown by the current study, the least number of galls were observed at neem/tobacco mixture at all 3 concentrations of 5g/L, 10g/L and 15g/L.

Neem has been used historically to rid the body of several forms of ecto-parasites and pests and the action is rapid. Hormone mimicking activities of neem extracts causes interference with the parasitic life cycle, inhibiting their ability to feed as well as prevent the hatching of eggs (Kumar and Navaratnam, 2013). Neem plant contents have been reported to be an effective bio-insecticide (Chary, 2009) and found useful in the control of many insect species of medical and veterinary importance.

Tobacco contains an active ingredient, nicotine, the substance acting as an insecticide. Tobacco contains 12 alkaloids and of these, nicotine is the most toxic (Singh and Singh, 1997). Nicotine causes synaptic poisoning. The nicotine mimics acetylcholine at the synapse. The receptor of the pest cannot distinguish between acetylcholine and nicotine (Chaube and Pundhir, 2005) and this result in acetylcholinesterase inhibition. The inhibition causes a build-up of acetylcholine and malfunction of the transmitting system (Chaube and Pundhir, 2005). Tobacco mixed with neem at 15g/L contains high levels of nicotine and azadirachtin respectively which are poisonous to *L. invasa.* This results in synergistic effect of both extracts' active ingredients in slowing down the life cycle of *L. invasa*.

The study also revealed that tobacco at 5g/L also had the most number of galls produced by the pest. Although the numbers of galls were low with the use of a higher concentration of tobacco (15g/L), they were still higher than the other two extracts namely neem and neem/tobacco mixture. In addition, high concentration of nicotine delays the hatching of eggs. Hatching of eggs also depends on the conditions necessary for hatching. Thus compared to the control, fewer eggs hatched which was equal to less number of galls developing but compared to neem extract and that of tobacco mixed with neem, nicotine effects were not fully exhibited as they worked better with those of azadirachtin for the latter extract. The use of lower levels of tobacco at 10g/L and 5g/L, showed a higher number of galls. This is due to the fact that low concentration of tobacco extract had less nicotine which allows hatching of the eggs.

Neem does not directly kill insects on the crop. It acts as an anti-feedant, repellent, egg-laying deterrent, protecting the crop from damage. The insects starve and die within a few days. Neem also suppresses the hatching of insect pests from their eggs. The current study also showed that each extract worked less effectively on its own compared to the mixture of the two namely neem and tobacco.

Adult Emergence

The neem extract at 15g/L showed the highest effectiveness by possibly lengthening the life cycle of the pest. This could be accounted by the fact that neem acts as an insect growth regulator, an effect of azadirachtin on *L. invasa.* The treated insect usually can't molt to its next life stage and dies. At lower concentrations of 10g/L and 5g/L, neem still reduced the number of adults that emerged than those of the other two extracts. This could be due to the fact that neem works well as a systemic pesticide and therefore disrupts the life cycle of the pest within the plant. Tobacco works better as a contact insecticide and therefore its mixture with neem reduces the effects of neem as the azadirachtin will be diluted by the nicotine thereby reducing its effects. However, the mixture of neem and tobacco at all 3 concentrations worked better than tobacco on its own at all concentrations as well.

In conclusion we can safely say that plants and their extracts are the future insecticides that control effectively or at least reduce effects of insect pests and yet are still safe to the soil ecosystem and to human beings. They are a cheap resolution to small scale farmers in developing countries especially the majority of African and Asian countries. If well studied and given a chance, they provide an enhanced alternative to the use of chemical control after stopping the use of some dependable fumigants. Farmers in developing countries can make use of Neem and Tobacco at 15g/L using both spraying and pouring as application methods in controlling *L. invasa* gall development and to suppress adult emergence, use of neem at 15g/L.

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