INSECTICIDAL ACTIVITY OF AZADIRACHTIN RELATED LIMONOIDS FROM CALLUS AND CELLS SUSPENSION BIOMASS EXTRACTS OF NEEM AGAINST JASSIDS, WHITEFLY AND THRIPS

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ABSTRACT

The efficacy of callus extracts (CE) and cells suspensions biomass extracts (SBE) of Neem was tested against three sucking insects of cotton including jassids (*Amrasca biguttula* Ishida), thrips (*Thrips tabaci*) and whitflies (*Bemisia tabaci* Genn). The callus and cells suspension cultures were raised on previously optimized MS medium supplemented with 1.0mg/L of BAP and 2,4-D and NAA. In different treatments, cotton leaves were sprayed with different dilutions (T1, T2, T3, T4, T5 and C) of callus extracts (CE) and cells suspensions biomass extracts (SBE) and test insects were infested in Petri dishes. The dilutions T1 (1:10 v/v extract: dist H_2O) and T2 (1:100 v/v extract: dist H_2O) of both CE and SBE showed highly significant results with 100% mortality rate after two days of infestation. The dilution T3 (1:1000 v/v extract: dist H_2O) also showed significant results with 64-94% mortality followed by T4 (1:10000 v/v extract: dist H_2O) with 28-80% mortality after five days of infestation. The dilution T5 (1:100000 v/v extract: dist H_2O) initially showed small mortality response and growth inhibition during first three days and then increase in insects population was noted on fourth and fifth days. Similarly 10–20% insects population was increased in negative control (C) treated with dist H_2O.

Key words: Biopesticide, Azadirachtin, Callus, Cell suspensions culture, biomass, Antifeedent, Mortality

Abbreviations: 2,4-D: 2,4-dichlorophenoxy acetic acid, BAP: 6-Benzylaminopurine, NAA: 1-Naphthaleneacetic acid

INTRODUCTION

The synthetic pesticides cause toxicological and environment problems such as insect resistance, killing of non-target fauna and accidental health hazards (Bakhetia *et al*., 1996), so the importance of use of alternative biopesticides is increasing. The Neem plant (*Azadirachta indica* A. Juss) contains more than 300 bioactive compounds including limonoids, phenolic acids, steroids and carotenoids (Kumar *et al*., 1996; Morgan and Wilson, 1999). Some of these bioactive compounds are insecticidally active in nature (National Research Council 1992; Govindachari *et al*., 1992). The extracts of Neem root bark, stem bark, leaves, fruits and seeds have anti-inflammatory,insect antifeedant, insecticidal, nematicidal, bactericidal and fungicidal activities (Satdive *et al*., 2001).

Azadirachtin is chemically a limonoid tetranortriterpenoid that has received attention as biopesticide due to its broad spectrum properties, low toxicity against non-target organisms and showed great potential against insects (Govindachari *et al*., 1992; Immaraju, 1998; Prakash *et al*., 2002). The antifeedant effects of azadirachtin have been reported against insects of various orders like Coleoptera, Diptera, Lepidoptera, Hemiptera, Homoptera, etc (Ascher, 1993; Mordue and Blackwell,

1993). Azadirachtin may affect hormonal system and metamorphosis program of insects. It seems to block the release of ecdysone hormones thus causing disturbance in molting, life cycle and insect's production (Schmutterer, 1990; Ascher, 1993). Azadirachtin possesses complex structure which cannot be synthesized chemically. Due to geographical factors (Yakkundi *et al*., 1995) and seasonal variation (Sidhu and Behl, 1996) azadirachtin contents in seeds vary significantly. Furthermore, the trees fail to grow in moderate climates and in frost. For utilization of azadirachtin in agriculture needs its continuous availability with standardized quality.

With increasing demand of this important biopesticide in constant amounts of standardized quality azadirachtin, plant cell cultures have been recognized as an alternative source for the production of azadirachtin round the year (Van der Esch et al, 1993; Prakash et al., 2002; Rafiq and Dahot, 2010). The plant cell culture technology provides a promising source of important secondary compounds which could not be produced through chemical synthesis. The occurrence of azadirachtin contents have been reported in calli obtained from bark, flower and leaf of Neem (Allan et al.,1994; Wewetzer, 1998; Veeresham *et al*., 1998; Kuruvilla *et al*., 1999; Rafiq and Dahot, 2010). The callus and cell suspension extracts of neem showed promising effect against *P. gossypiella*, *H. armigera* and *S. litura* (Rafiq *et al*., 2012).

Amrasca devastans, *Bemisia tabaci* and *Thrips tabaci* are important insect pests of cotton crop that directly affect and reduce the cotton production by sucking transmitting different diseases (Ruscoe *et al*., 1996). Keeping in view various applications of azadirachtin related limonoids, the efficacy of callus and cells suspension extracts of neem were assessed against the sucking insects of cotton including jassids (*Amrasca biguttula* Ishida), thrips (*Thrips tabaci*) and whitflies (*Bemisia tabaci* Genn).

MATERIALS AND METHODS

Callus and Cell Suspension Cultures: For callus induction, immature flowers were collected from a ten years old Neem plant growing at University of Sindh, Jamshoro campus, sterilized with NaOCl and first inoculated for 15 days on previously optimized MS medium (Murashige and Skoog, 1962) additionally supplemented with 9% sucrose, 1 mg/L BAP, 1 mg/L 2,4-D, 0.2 mg/L NAA and solidified with 0.3% phytagel and then subcultured on same medium with 3% sucrose. The cultures were incubated in growth room at 25±2°C with 14/8 hours light/dark photoperiod. For initiation of cells suspension cultures, yellow to light brown proliferating calli were crushed, sieved and inoculated in 250 mL flask containing 50 mL MS liquid medium supplemented with 1 mg/L 2, 4-D, 1 mg/L BAP, 0.2mg/L NAA and 3% sucrose and incubated in shaking incubator at 105 rpm and 25±2°C with 14/8 hours light/dark photoperiod. The homogenous cells suspensions were obtained through six times subculturing in the same media after every seven days interval.

Isolation and Determination of Azadirachtin -related Limonoids: For isolation of azadirachtin related limonoids, callus and filtered cells suspensions were dried separately on filter paper at 40°C for 72 hours. 20 gram of each callus and cells suspension dried samples were ground in pestle and mortar in 10 ml methanol, extracts were centrifuged at 8000 rpm and 4°C, supernatant was collected and subjected to extraction

twice. The methanol was evaporated in 40°C in water bath and the remaining crude extract was dissolved in 20 ml of distilled water and azadirachtin related limonoids were quantified through spectrophotometer following Dai et al., (1999) method; finally the crude extracts were adjusted to 1 mg/ml.

Laboratory Scale Insect Bioassays: For insect bioassays, five dilutions of both Neem callus extract (CE) and Neem cells suspension extract (SE) were prepared with dist H_2O separately with following ratios; T1 (1:10 v/v extract: dist H_2O), T2 (1:100 v/v extract: dist H_2O), T3 (1:1000 v/v extract: dist H_2O), T4 (1:10000 v/v extract: dist H_2O), T5 (1:100000 v/v extract: dist H_2O) and C (negative control) only dist H_2O. The efficacy of different preparations CE and SE was assessed against three sucking cotton insects including jassids (*Amrasca biguttula* Ishida), thrips (*Thrips tabaci*) and whitflies (*Bemisia tabaci* Genn). All the sucking insects were collected during July to September, 2010 from cotton field at Nuclear Institute of Agriculture (NIA), Tandojam, Sindh, Pakistan.

For bioassay, medium sized fresh leaves of cotton were collected from field, washed with distilled water and blotted dry with tissue paper. The leaves were either cut into two pieces containing midrib or used in full, sprayed with different preparations (T1, T2, T3, T4 and T5) of callus extracts (CE) and cells suspension extracts (SE) and placed in Petri dishes. For negative control, leaves were sprayed with distilled water only. Twenty five sucking insects of each jassids, thrips and whitflies were infested separately on treated leaves in each Petri dish with two Petri dishes for each preparation. The experiment was performed two times in the same way thus one hundred insects of each species were infested for each preparation. The experimental Petri dishes were incubated at 30±2°C with 80-90% relative humidity in low light intensity and data was recorded every 24 hours for 5 days. The mortality of insects was calculated by determining the percentage of dead insects based on the following formula:

$$\text{Percentage Mortality} = \frac{\text{Number of dead insects/experiment}}{\text{Total number of insects/experiment}} \times 100$$

The experiments were repeated three times and mean value was taken. The data was statistically evaluated to determine the standard deviation (SD) of the mean.

RESULTS

Different treatments (from T1 to T5) of callus extracts (CE) and cells suspension extracts (SE) were used for bioassays against three sucking insects including cotton jassid (*Amrasca biguttula* Ishida), whitefly (*Bemisia tabaci* (Gennadius), thrips (*Thrips tabaci*). The mortality rate of three treatments T1 and T2 of both CE and SE were highly significant and all insects were found dead from all three species after during three to four days of infestation. As jassids were infested on leaves treated with different preparations of both CE and SE, 100% mortality was observed within two and three days in preparations T1 and T2, followed by 78-80% and 40-44% mortality after five days in T3 and T4 respectively. In case of preparations T5 of both CE and SE, 16-22% mortality (78-84% survival of insects) was recorded in first three days

than an increase of insects population was observed as during 4th and 5th day of infestation. Similarly 20% increase in insects population (120 insects) was also recorded after five days in Petri dishes of negative control (C) treated with distilled water (Figure-1).

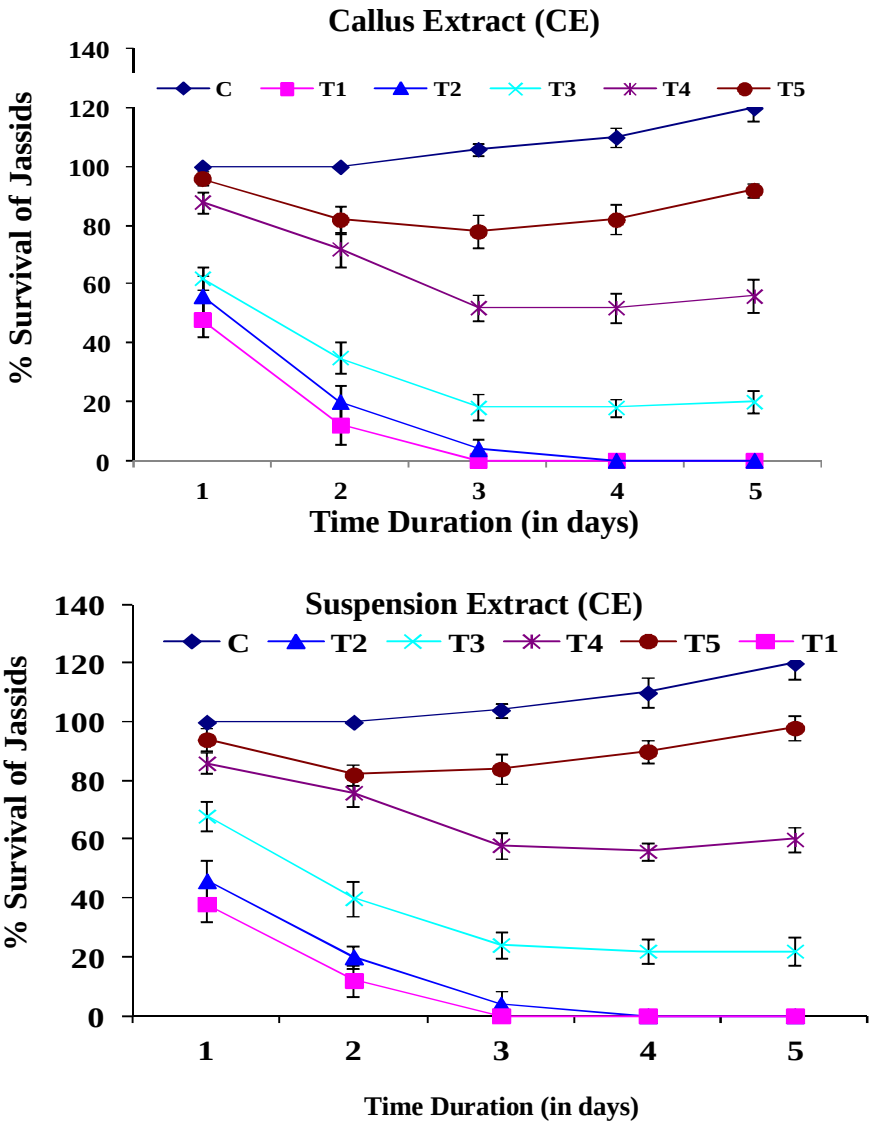

Figure-1: Effect of various Preparations of callus and cell suspension extracts of neem on mortality of jassids (*Amrasca biguttula* Ishida) (Mean±S.D)

Whiteflies were also infested on treated leaves by different dilutions (T1 to T5) of CE and SE that showed variable response of mortality and insect population growth. The mortality rate was recorded higher (100%) as all whiteflies were found dead during 2–3 days of infestation in treatments with higher concentrations of extracts (T1 and T2) of both samples CE and SE. A decrease in mortality rate was recorded within the preparations T3 (1:1000 v/v extract: dist H_2O) of CE and SE with 78% and 64% mortality after five days of infestation. The mortality rate was further decreased to 40% and 42% as 60–62% insects were alive in preparations T4 of CE and SE respectively. The bio-toxicity response of preparation T5 (1:100000 v/v extract: dist H_2O) of CE and SE was insignificant with 78 – 84% survival of insects (16 – 22% mortality) in first three days and then insects population was increased with 88 and 104 insects were recorded in preparations T5 of CE and SE. The insects populations were also significantly increased from 100 to 114 after five days in negative control (C) treated with distilled water (Figure-2).

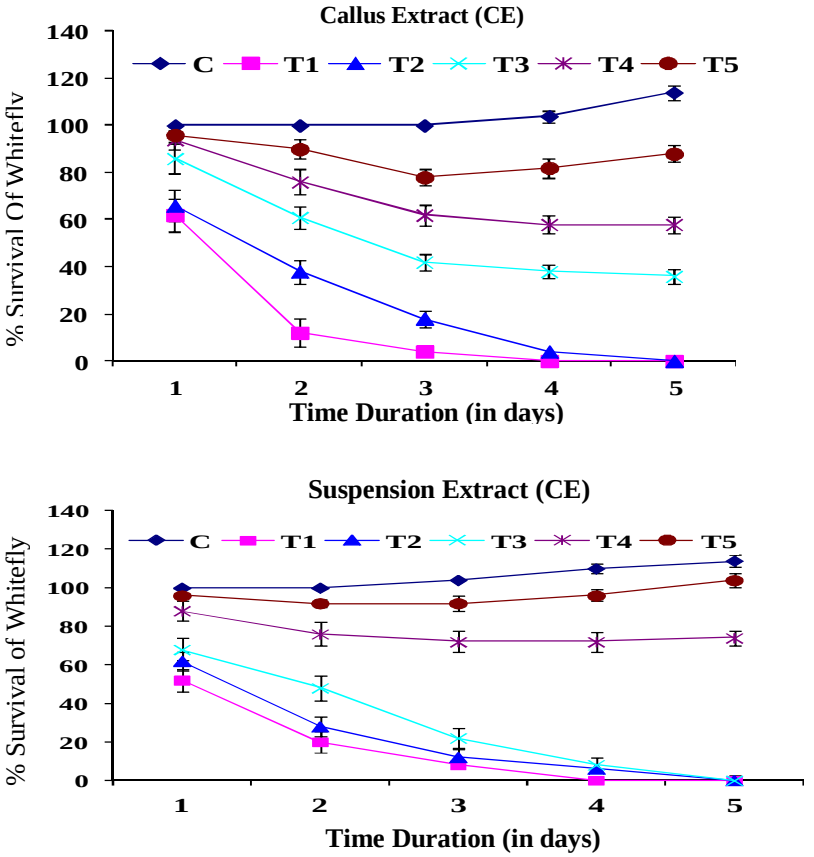

Figure-2: Effect of various Preparations of callus and cell suspension extracts of neem on mortality of Whitefly (*Bemisia tabaci* Genn) (Mean±S.D)

The preparations T1 and T2 of both CE and SE also showed 100% morality against thrips during first two to three days of infestation. The preparations T3 of both CE and SE also showed significant mortality with only 6% and 8% survival of insects respectively after five days of infestation. The mortality rate was decreased as extracts were further diluted in preparations T4 (1:10000 v/v extract: dist H_2O) and T5 (1:100000 v/v extract: dist H_2O) of both CE and SE while the population of thrips was significantly increased from 100 to 110 in number after five days of infestation in negative control (C) treated with distilled water (Figure-3). According to data obtained, both callus extracts (CE) and suspension cells extracts (SE) based preparations showed bio-toxicity against all three sucking insects including jassid, whitefly and thrips. Among all preparations, T1 and T2 showed the highest mortality response followed by good bioactivity response (T3), with lower (T4) and the lowest efficacy against tested insects (T5) (Figures 1-3)

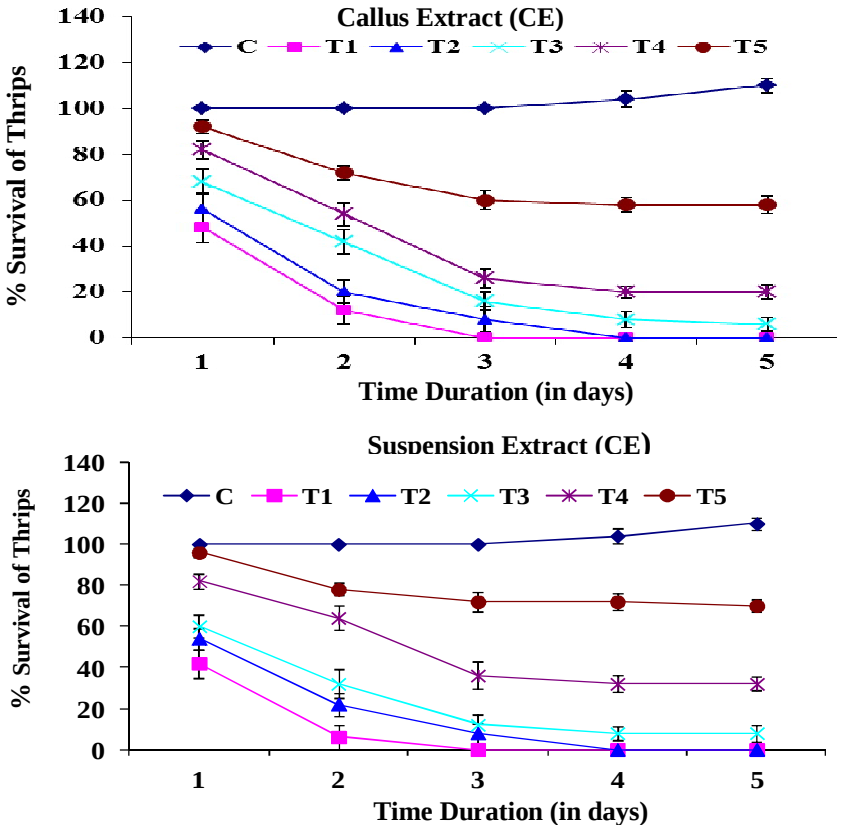

Figure-3: Effect of various Preparations of callus and cells suspension extracts of neem on mortality of Thrips (*Thrips tabaci*) (Mean±S.D)

DISCUSSION

The efficacy of extracts of various parts of Neem against insects has also been described. Azadirachtin control inhibit the growth of insects of different orders Coleoptera, Diptera, Lepidoptera and Hemiptera including aphids, thrips, whiteflies, mealybugs, leaf worms, pink bollworms, armyworms, caterpillars, flies, moths, fungus gnats etc. (Ascher, 1993; Mordue and Blackwell,1993). Azadirachtin may either limit the insects feeding leading to insect death (Thomson, 1992) or may influence hormonal system that modifies the metamorphosis program of insects (Schmutterer,1990; Ascher, 1993). The preparations of T1 (1:10 v/v extract: dist H_2O) and T2 (1:100 v/v extract: dist H_2O) of Neem callus extracts (CE) and cells suspension biomass extracts (SBE) significantly reduced the populations with 100% mortality of all test insects whiteflies, thrips and jassids up to two days. Further dilutions of preparation T3 (1:1000 v/v extract: dist H_2O) of CE and SE also reduced and deterred the population of test insects with 20-36% survival of jassids, 36% survival of whiteflies in CE and 6-8% survival of thrips after five days of infestation. The results showed that as dilutions of extracts were increased in preparations T4 (1:10000 v/v extract: dist H_2O) and T5 (1:100000 v/v extract: dist H_2O), it increased the survival of insect population and reduced the mortality of the test population.

The biological toxicity of Neem seeds extracts and Neem oil against insects has also been reported by other researchers. Lowery and Isman (1994) also reported 94-100% mortality of second instar nymphs of currant lettuce aphid and green peach aphid after nine days when treated the leaf discs with 1.0% Neem seed oil. During semi-field trials for the assessment of biological activity of Neem seeds products against the whitefly, Dimetry et al., (1996) reported the reduction of population density of adult whiteflies due to different treatments after one hour as compared to control. Weathersbee and Mckenzie (2005) assessed that 4.5% azadirachtin containing Neem based biopesticide against *Diaphorina citri* Kuwayama, showed significant repellent effect from treated plants. Azadirachtin (10ppm concentration) also showed developmental inhibition and ultimately death of all *Diaphorina citri* within seven days. Nathan et al., (2005) observed the bioactivity of five limonoids of Neem including azadirachtin, deacetylnimbin, deacetylgedunin, gedunin and salannin on *Anopheles* mosquitoes and 100% larvae mortality was recorded with 1.0ppm concentration of azadirachtin. Gupta and Sharma (1997) found that Neem oil and Neem seed extracts significantly reduced the adult and nymphal populations of *Bemisia tabaci*. Khan et al., (2002) demonstrated that due to the antifeedant and deterrent effect of Neem extracts, the populations of jassids, thrips and whiteflies are forced to leave the locality. Khattak et al., (2006) also reported that 2% Neem oil and 3% Neem seed extracts significantly reduced the populations of jassids, thirips and whiteflies of cotton. The reduction in population of test insects after 24 hours of spray may be due to antifeedent and deterrent effect of Neem extracts which forced the insects to leave the locality.

Similarly the antifeedancy of callus and suspension cells has been studied against desert locust (Kearney et al., 1994). Rafiq et al., (2012) also reported the efficacy of callus and suspension cells extracts of Neem against lepidopteron

insects. The mortality rate of leafworm, cotton boll-worm and pink bollworm was found significantly higher at higher concen-trations of callus and cell suspension extracts of Neem.

Conclusion: Neem oil and Neem extracts from various parts of intact plants like seed, flower, leaf, bark etc. has been reported to be used as biopesticides. In present study, callus extracts and cells suspension extracts of Neem were evaluated for insecticidal activity against three sucking insects of cotton. The preparations T1 and T2 of both callus extracts (CE) and cell suspension extracts (SE) showed 100% mortality response against jassids, whiteflies and thrips. The preparations T3 and T4 with water dilutions also reduced the population of test insects significantly.

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