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Degradation of azadirachtin A on treated maize and cowpea and the persistence of *Azadirachta indica* seed oil on *Callosobruchus maculatus* and *Sitophilus zeamais*

Katamssadan H. Tofel [a, b, *], Elias N. Nukenine [c], Matthias Stähler [a], Cornel Adler [a]

[a] Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Ecochemistry, Plant Analysis and Stored Product Protection, Königin-Luise-Str. 19, D-14195, Berlin, Germany
[b] Department of Biological Sciences, Faculty of Science, University of Bamenda, P.O. Box 39, Bamenda, Cameroon
[c] Department of Biological Sciences, Faculty of Science, University of Ngaoundere, P.O. Box 454, Ngaoundere, Cameroon

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ABSTRACT

Azadirachta indica seed oil has long been used in many parts of the world for the control of various insect pests. The quantification of its known insecticidal compound Azadirachtin A on treated commodities remains a challenge. The degradation of Azadirachtin A in treated cowpea and maize was determined with HPLC-MS as well as the toxicity of *A. indica* seed oil on their respective major pests is storage between 0 and 180 days. Azadirachtin A degraded slowly on treated maize from 1.31 mg/kg (0-day) to 0.38 mg/kg (180-day) while on cowpea it degraded from 1.14 mg/kg (0-day) to 0.43 mg/kg (180-day). *A. indica* oil caused a significant day-dependent mortality of adults *Callosobruchus maculatus* and *Sitophilus zeamais* and its effectiveness decreased with time. The tested oil was more persistent for inhibiting progeny production than on adult mortality. Further studies are needed to evaluate the quality of treated grains at different storage times.

1. 1- Introduction

Azadirachta indica A. Juss (Meliaceae) commonly called neem is one of the remarkable plant studied by several researchers for its insecticidal and medicinal activities. It is considered as the plant of plant of the 21st century (Ilesanmi and Gungula, 2013) and the popularity of its products is increasing day by day. Products from leaves, barks and seeds of this tree have been used for their medicinal properties (Sidhu et al., 2003; Nandagopal and Ghewande, 2004). Neem seed oil is used for soap manufacture (Schmutterer, 1990), motor lubricant and biodiesel and an efficacious insecticide (Girish and Shankara, 2008). Barks and leaves of this plant are employed for the treatment of some diseases and are good antidotes against snake bite and scorpion sting (Yengué and Callot, 2002). The twigs of neem tree are used for dental hygiene (Agrawal, 2002). It is toxic to over 500 insect species (Schmutterer, 1990; Athanassiou et al., 2005; Kavallieratos et al., 2007; Roy et al., 2010) including stored product insect pests of cowpea and maize (Bélanger and Musabyinama, 2005; Iloba and Ekrakene, 2006; Debashri and Tamal, 2012). To ensure food security for the whole year, farmers store more than 75% of their harvested cowpea and maize (Kumar, 1991) and therefore they have to protect these grains which are heavily damaged respectively by the cowpea weevil *Callosobruchus maculatus* Fabricius and the maize weevil *Sitophilus zeamais* Motschulsky. Neem oil is reported to protect stored products up to five or six months when applied at the rate of 1% (Guet, 2002). The high quantity of neem oil applied to grains gives a bitter taste to products and therefore not accepted by local farmers. To promote the use of safer *A. indica* neem seed oil combined with good persistence in stored product protection, the application of less than 1% of oil content needs to be reconsidered. Such studies could decipher the lower dosage of oil, and thus help growers to obtain more efficient plant-based insecticidal product for stored product protection with a minimized bitter effect. The main ingredient in neem products known for its efficacy against insect pests is Azadirachtin A (Schmutterer, 1995) which is extremely

* Corresponding author. Department of Biological Sciences, Faculty of Science, University of Bamenda, P.O. Box 39, Bamenda, Cameroon.
E-mail address: tofelhama@yahoo.fr (K.H. Tofel).

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labile in light (Johnson et al., 2003) and has it potential efficacy within 10 and 12 days when used properly (Guet, 2002). Farmers in sub-Saharan Africa treat and store their products for about one year. If the amount of the active ingredient is not enough to protect the treated grains after certain time, this could lead to high insect population growth that may endanger the stored products. Systematic scientific experimentation is necessary to determine the quantity of Azadirachtin A found in treated cowpea and maize and stored for different periods as well as the persistence of *A. indica* seed oil on treated cowpea and maize against *C. maculatus* and *S. zeamais* respectively. This is the first study reporting the quantification of Azadirachtin A on treated grains with neem oil at different storage periods.

2. Materials and methods

2.1. Collection of Azadirachta indica seeds and oil extraction

Ripe seeds (de-pulped by birds) were collected on the ground under *A. indica* trees in the Meskine quarter (latitude 10°33.16′ N, longitude 14°815.04' E and altitude of 356 m.a.s.l.) of Maroua, Far-North region, Cameroon in May 2011. The city of Maroua is in the Sudano-Sahelian agro-ecological zone (IRAD, 2007). This agro-ecology is characterized by two seasons: wet (June to September) and dry (October to May). Annual rainfall ranges between 800 and 1000 mm. Annual mean temperature is 29 °C, with a maximum of 39 °C in March and minimum of 17 °C in January. Average annual r.h. stands at 67%.

The collected seeds were dehusked and sun-dried. The drying temperature of the kernels was 34 ± 4 °C for seven days. The dried kernels were stored in a deep-freezer at −14 °C, until transported to Julius Kühn-Institut (JKI) Berlin, Germany (after 4 months). The extraction of the oil was carried out using a mechanical press (CA59G Komet, Mönchengladbach, Germany). Two kilograms kernels were introduced into the press and crude neem oils were collected, filtered and kept in opaque glass.

2.2. Origin of cowpea and maize

The maize variety was yellow Ricardino (KWS) harvested in an experimental field of Julius Kühn-Institut (JKI), Braunschweig, Germany in 2012. The organic cowpea (Black-eyed bean, Perou variety) was purchased in a tropical food store in Berlin, Germany.

2.3. Insects

Sitophilus zeamais was reared on maize and *C. maculatus* on cowpea in controlled temperature and humidity chambers (25 ± 1 °C and 60± 3% r.h.) in darkness. Adults of *S. zeamais* and *C. maculatus* were obtained from laboratory colony kept since 1968 and 2011 respectively at the Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection (Julius Kühn-Institut), Berlin, Germany. Insects aged 1 day old for *C. maculatus* and between 7 and 14 days for *S. zeamais* were used for all bioassays with cowpea and maize as substrates, respectively.

2.4. Azadirachtin A determination on treated cowpea and maize

The volumes of 0.1, 0.15, 0.2, 0.25 and 0.3 mL of neem seed oil were separately pipetted into 50 g of maize or cowpea contained in 250 mL glass jars to give the concentrations of 2, 3, 4, 5 and 6 mL/kg of maize or cowpea. Untreated grains were considered as control. A sample 5 g of grain was taken at 0, 1, 3, 7, 10, 14, 21, 30, 60, 90, 120, 150 and 180 days after treatment for azadirachtin A determination. The 5 g of cowpea or maize were weighed into a 50 mL polypropylene centrifuge tube and 100 μl of surrogate (spinosyn A 100 g/L) were added. Extraction was performed by adding 25 mL acetone/water in proportion 80:20 v/v. The mixture was shaken using an ultrasonic bath for 15 min and then vortex-mixer for 45 min. An aliquot of 500 μl from the upper layer of extract was transferred to an Agilent vial and then dried to evaporate water. The extract was diluted with 1 mL of methanol/water 1:1 (v/v) containing an internal standard spinosyn L (used for quantification) at the concentration of 25 pg/μL and subsequently kept in dark at 4 °C until analyze via LC/MS/MS. Each treatment was replicated four times and for each tube two replications was done for a total of eight repetitions.

Liquid chromatography–electrospray ionization-tandem mass spectrometry, in positive ion mode, was used to separate, identify, and quantify azadirachtin A. For the LC analysis, a Shimadzu Prominence UFLCXR HPLC system (Agilent Technologies) with a binary pump was used. The analytical column employed was a reversed-phase C18 of 50 × 3 mm and 2.6 μm particle sizes. The mobile phase A was methanol-water (90:10, v/v) with 0.1% acetic acid + 5 mmol Ammonium acetate. The mobile phase B was water with 0.1% acetic acid +5 mmol Ammonium acetate. The gradient program started with 0% of A, constant for 2 min, followed by a linear gradient up to 100% A in 3.5 min, and finishing with 100% A constant for 3.5 min. After this 5.5 min run time, 3.5 min of post-time followed using the initial 30% of B. The flow rate was set constant at 0.9 mL/min during the whole process, and the injection volume was 5 μL. For the mass spectrometric analysis, a AB SCIEX QTRAP 4000 MS/MS system (AB Sciex Instruments) was used, equipped with a turbo ion spray source operating in positive ionization mode, set with the following parameters: Ion Spray (IS) voltage: 5500 V; curtain gas: 20 psi; nebulizer gas (GS1): 70 psi; auxiliary gas (GS2): 50 psi; source temperature: 550 °C. Nitrogen was used as the nebulizer and collision gas. Optimization of the compound was performed by flow injection analysis (FIA), injecting individual standard solutions directly into the source. AB SCIEX Analyst software 1.5.2 was used for data acquisition and processing.

2.5. Adult toxicity test and F_1 progeny production

Similar dosages of oil as applied for the degradation of azadirachtin A bioassay described above were used for this assay. Controls consisted of grains without neem seed oil. Each jar (250 mL) was shaken with a bidimensional mixer (Gerhardt, Dreieich, Germany) for approximately 4 min to ensure uniform distribution of the oils to the entire grain mass. To assess the persistence of the treatments, 20 adult beetles (*S. zeamais* or *C. maculatus*) were exposed to treated grain (maize or cowpea) which had been stored for 0, 15, 30, 60 and 180 days. Mortality counts were carried out 3 and 5 days after exposure for *C. maculatus* and *S. zeamais* respectively. Control glass jars also separately received twenty insects each. All treatments were arranged in a completely randomized design on shelves in the laboratory (25 ± 1 °C and 60± 3% r.h.) and each treatment had four replications. Insects were considered dead when no movement was observed after touching them carefully with forceps. After the 3-day and 5-day mortality recordings respectively for *C. maculatus* and *S. zeamais*, all the insects were separated from the grains and discarded. The grains were left inside the jars and all F_1 progeny were counted (Nukenine et al., 2007). To avoid generation overlaps, F_1 progeny were recorded 40 days and 50 days after infestation for *C. maculatus* and *S. zeamais* respectively.

2.6. Data analysis

Data on the amount Azadirachtin A and the number of progeny