Nanofibers as a Vehicle for the Synthetic Attactant TRIMEDLURE to be Used for *Ceratitis capitata* Wied: (Diptera, Tethritidae) Capture

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Abstract: The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wied.) is considered a serious pest of citrus fruits in the southeast of Brazil because of the direct economic impact they have on fruit production and quarantine restrictions for fruit exports. Monitoring and detection of the medfly, using food bait and synthetic attractants, is a key step towards signalling the need for management and control. The aim of this study has been to verify the attractiveness of innovative nanofiber formulations with Trimedlure (TML) for the male of *C. capitata* in laboratory and field cage tests.

Material and Methods: The nanofibers were produced by solution or emulsion electrospinning, containing TML and polymers, such as polycaprolactone, PEG-polycaprolactone, ethyl cellulose and polyvinyl acetate-PVP.

Results: At the laboratory the electrophysiological responses were accessed by the eletroanntenogram technique and in the field by the cage test. The bioactivity of *C. capitata* antennae was highest when stimulated with all TML nanofiber treatments rather than their controls. There were no differences among the TML nanofiber treatments. In the field cages the same number of medflies were found on the adhesive traps baited with one of each of the TML nanofibers.

Keywords: Medfly, electrospinning, nanofibers, pheromone dispenser.

INTRODUCTION

The Mediterranean fruit fly *Ceratitis capitata* Wied. is one of the major pests of economic significance in the world [1]. It is widely distributed throughout Brazil and is considered a pest for fruit production in the country [2]. Besides infesting a large range of fruit, it limits the Brazilian fruit exports because of quarantine restrictions [3]. According to Jang and colleagues (2005), the threat of the establishment of the Mediterranean fruit fly has always been of concern for states and countries involved in the international fruit trade, which stimulates a constant search for new methods of detection and control based on the use of semiochemicals [1]. According to Beroza and coworkers (1961) the trimedlure was developed from an intensive testing programme by the United States Department of Agriculture (USDA) to be used as the standard synthetic attractive for *C. capitata* males [4]. The trimedlure is used commercially in formulations which provide controlled release of active ingredients for up to eight weeks [1].

The pheromone compounds attract pests to the crops and are used for insect control techniques. Their use in integrated pest management (IPM) is fundamental because of their agronomical efficiency and being environmentally friendly. However, by being highly volatile, they lose their attractive effects fast, so a formulation that gives a delayed delivery is desirable. In the search for new and innovative dispensers for crops, micro and nanotechnology is a potential and promising candidate. Hellman and colleagues (2009) pioneered the use of electrospun formed nanofibers for pheromone dispenser development [5].

In Brazil, a pioneering and innovative alternative has emerged which involves the use of nanotechnology for the production of semiochemical and pheromone dispensers, providing a controlled release of the volatile compound associated with a renewable source and more efficiency or matrice biodegradability of the dispensers. Jacobs & Mason (1993) reported that controlled release may increase the efficacy and specificity of the agent, reducing both the required doses and the risk of harm to the environment [6]. An important feature for use of pesticides in agriculture is the biodegradability of these materials, which may favour the use of these vehicles in the controlled release of pheromones [7].

In Germany, Hellman and coworkers (2009) conducted pioneering studies using nanofibers as vehicles for semiochemicals of agricultural interest [5].

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The authors used the technique of electrospinning to produce renewable source polyamide and cellulose acetate nanofibers, containing the synthetic sex pheromone of the grape moth *Lobesia botrana* ([Denis & Schiffermuller]) (Lepidoptera, Tortricidae) and later used them in bioassays under field conditions. The experimental results showed the efficiency of the nanofibers in releasing the pheromone of the grape moth, thus enabling the management of this pest in grapevines.

Developing an Innovative Pheromone Dispenser

Due to their higher surface area to volume ratio, nanofibers have become the object of study for drug delivery [8]. Electrospinning is one of the most interesting methods for drug delivery and the nanofibers produced can also bring benefits to the area of agriculture [9-11]. The volatile organic compounds (VOC), such as those emitted by plants or even insects, like pheromone and kairomone, are especially susceptible to sunlight exposure, leading to photocatalyctic degradation of the incorporated product. A polymeric protection, therefore and also UV protection in the form of polymer additives, are fundamental for the protection of the agrochemical to be sprayed or deposited in the fields. Electrospinning and electrospraying are novel techniques used experimentally for making polymeric nano or microscale fibers or particles, respectively, for a variety of applications.

Polymer Selection

For agricultural use, elastomeric polymers are a common dispersing system for pheromone release in the form of rubber or silicone dispensers. The polyethylene tubes are also commonly used because of their delayed and constant release. They are not degradable polymers and do not stem from a renewable source, which would be preferable for dispersion in the environment. Therefore, they need to be collected from the place where they are deposited. There are some polymers which are commonly used in polymeric matrix drug release for pharmaceutical purposes; both synthetic and renewable sources [12]. Some of the materials most frequently used in pharmaceutical controlled release include hydroxypropyl methylcellulose (HPMC), ethyl cellulose (EC), polyvinylpyrrolidone (PVP) and polyacrylic esters. Aliphatic polyesters have been employed as slow-release agents in agriculture for a long time [13]. They degrade into natural materials and are harmless to the environment while slowly releasing the encapsulated herbicides or pesticides. The cellulose derivatives, both esters and ether derivatives, are also from renewable sources. Polyvinylpyrrolidone are used with these polymers for introducing an amphiphilic character for these blends, which is desirable for controlled release purposes.

Based on previous studies made by the present group, some polymers were selected for controlled release of pheromone in the field. The polymers selected for pheromone dispenser making were polycaprolactone (PCL) and ethyl cellulose (EC) polymers because of the renewable source and known application as controlled release vehicles, plus their feasibility for employing the electrospinning technique. The following amphiphilic blends of polycaprolactone associated with polyethylene glycol (PEG- PCL) and the blend of polyvinyl acetate- vinyl pyrrolidone (PVAc-PVP) were selected as vehicles for the synthetic para-pheromone TML of medfly *C. capitata*.

The present group are Brazilian pioneers in the use of nanofibers as a vehicle for releasing semiochemicals of agricultural interest. Given this scenario, the objective of this study has been to develop and evaluate nanofibers made from different polymers. The attractiveness of innovative nanofiber formulations with Trimedlure (TML) for male of *C. capitata* were evaluated in laboratory and field cage tests.

MATERIALS AND METHODS

The nanofibers were produced by solution or emulsion electrospinning containing TML (Policore Trimedlure AgriSense) as a medfly attractant with different polymers and their blends. Electrospinning is a technique focused on nanofiber or small scale particle production, which can use a variety of polymers. The polymers used were: Polyvinyl acetate, molecular weight: 500,000; Sigma-Aldrich; Poly (ε-caprolactone) (PCL), molecular weight: 70,000-90,000; Sigma-Aldrich; Ethylcellulose (EC), viscosity 4 cPs (5% solution in 80/20 toluene/ ethanol), ethoxy-content 49.5 %: Sigma-Aldrich. The amount of the active ingredient in the electrospinning solution was 55% (550 microliters to 10 mls of solution).

The EC and PCL particles were produced by electrospraying, while the PEG- PCL, PVAc- PVP were produced by the electrospinning technique. The polymers, para-pheromone TML and oil phase were dissolved in organic solvents and mixed with the water phase containing the surfactant and submitted to the electrospinning machine. The organic solvents used for the solution as emulsions or dispersions were acetone, ethanol, chloroform and tetrahydrofuran, all reagent grades. When one solvent was not able to produce spinnability or sprayability, a co-solvent was selected among the same organic solvents.

The polymer or its blend were dissolved using the solvents for obtaining a final 0.02 to 10% (w/v) concentration of TML. The solution was fed in to the electrospinning machine with a multi-jet syringe pump at a rate of 0.05 ml/min, using a 3 ml syringe with a blunt ended needle (BD Precision Glide 22G). A voltage of 1.81 kV/cm was applied between the needle and the collector. A nanofiber film was deposited on the collec-tor to cover the glass slides and directly onto the metal stubs when the intended use was for SEM analysis.

Electrospinning Apparatus

A custom made electrospinning machine was used. A Matsusada Precision Inc 60 kV HV supply powered the machine. A syringe pump was designed for research purposes and consisted of 3 mL syringes and a rotary aluminum collector adjusted to about 60 rpm, inside a Permution™ CE0730 Evolution model fume hood.

The polymeric solution concentration was sufficient to begin making the fibers or particles intended for each case, varying between 5 % to 15% of the weight of the solution. The shape of the fibers or particles was first assessed in an optical microscope and then analysed in a SEM (JEOL JSM 6060).

Para-Pheromone Trimedlure Fruit Fly *C. capitata*

As a model study used for the Mediterranean fruit-fly capture, the para-pheromone trimedlure (TML) was selected, which is already an established attractive.

To assess the release of the TML compounds from the different nanofibers, the electroantennography technique was used in order to verify the volatile perception of the *C. capitata* antennae males following the stimuli, using different formulations containing the attractant. Subsequently, the nanofibers which produced the largest electrophysiological responses were selected for use in bioassays for evaluating the efficiency of the attractant for flies in terms of the field cage.

Insects

The *C. capitata* individuals used in the bioassays were obtained from rearing at BIOECOLAB-UFRGS, using the methodology proposed by Teran (1976) with adaptations. Male *C. capitata,* aged 20 to 30 days, were used in the bioassays.

Electroantennographic Bioassays

The cases and controls were evaluated by electroantennographic bioassays. The cases were the different polymeric formulations incorporating the trimedlure (TML) attractant, while the controls consisted of the same nanofibers without incorporated trimedlure. Each fly used in the tests was observed with a stereomicroscope (75 X magnification), with a section made in one of the antenna at the pedicel, which was positioned in a bifurcated silver electrode. The basal and apical ends of the antenna were attached to the recorder and the neutral electrode, respectively. Both ends were immersed in salt-free gel Spectra 360 for the electrical conductivity of the sample. The analog response signal, in millivolts, was captured, amplified and processed with a IDAC-4 (Syntech®) data acquisition controller and subsequently recorded by EAG2000 software (Syntech®). To prevent drying, thus increasing durability of the antenna, the latter were kept moist throughout the tests. To achieve this, an air current, directed to the antenna, passed through an Erlenmeyer frask (50 ml) containing distilled water. The portions of nanofibers (2cm x 2cm) containing trimedlure and their control, were individually placed inside Pasteur pipettes. The antennae were subjected to air pulses generated by a flow controller in a volume of 2.5 mL / 0.5 s with different treatments. The antennae were stimulated by placing the front end of the pipette into a hole in the wall of a metal tube (1cm diameter x 18cm length) oriented towards the antennae, followed by a second air pulse. A period of one-minute between successive stimuli was provided for the antennae to regain their odour perception ability. The electrophysiological responses of each portion of the nanofibers, in millivolts, were recorded and compared statistically. The antennae were used only once in each repetition. Ten *C. capitata* males of 20 to 30 days of age were used for each test response.

Field Cage Conditions Bioassays

Two 190 x 190 x 200 cm cages, lined with voile fabric and with access to the interior *via* a door were used to assess the attractiveness of different

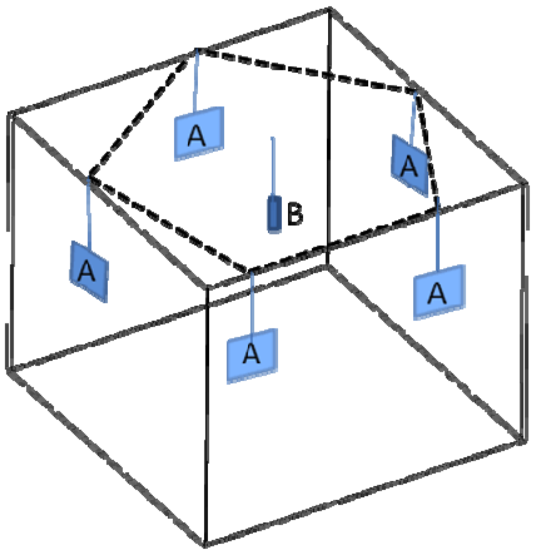

Figure 1: Schematic representation of the semi-field conditions bioassays cage used for evaluating the trimedlure nanofiber attractiveness for the *Ceratitis capitata* fruit-fly. A-adhesive plates containing the TML attractant and control; B-vial containing fly food.

formulations of nanofibers containing the fruit-fly attractant. At the inside top of each cage five points were made, which joined to one another. In the smallest gap between them, a pentagon was formed with 120cm sides where the four treatments and the control were placed (Figure **1**). Four adhesive plates with dimensions of 9cm x 9cm were baited with nanofibers with dimensions of 2cm x 2cm, each of them with one of the treatments, which served as a trap to catch the flies. The control was performed with an inert adhesive plate. The inert adhesive plate did not contain trimedlure and was not expected to attract flies. The objective of this test was to verify if the flies had a preference for some of the formulations containing the TML attractant. Each plate was hung in one of the vertices of the pentagon, at a distance of 20 cm below the ceilings of the cages. The treatments were rotated in a clockwise direction so that each of them occupied a different position within the cage in each of the 10 replications. A bottle containing 10 ml solution of 10% honey was hung in the centre of the cage, 20 cm from the ceiling, which served as food for the flies. In each replicate, 50 *C. capitata* males, aged between 20 and 30 days, were released into the cages. After 24 H the number of flies caught on each sticky card was recorded and the data was compared statistically (ANOVA and/or Kruskal-Wallis test at 5% significance level).

RESULTS

Electroantennographic bioassay results indicated that the different formulations of nanofibers containing the TML triggered electrophysiological responses significantly higher than their respective controls (ANOVA, $P < 0.05$, $n = 20$). Only the stimulus from the blend EC-PVAc nanofibers produced lower responses than the other TML treatments (Figure **2**). The higher antennal bioactivity obtained in the treatments containing TML shows the presence and release of volatiles by the nanofibers.

In the field cage conditions bioassay, a significantly greater number of taps *C. capitata* were found adhered

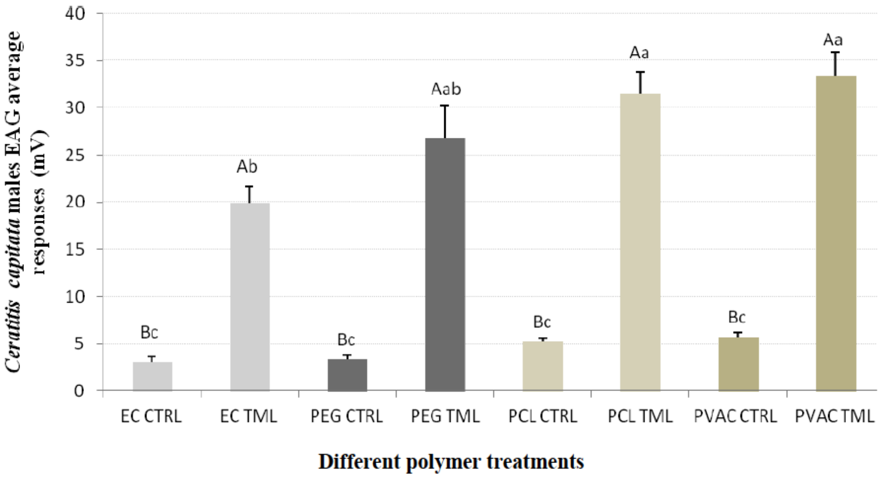

Figure 2: *Ceratitis capitata* male electrophysiological (EAG) average responses of the nanofibers containing the Trimedlure (TML) attractant made from EC-PVAc, amphiphilic PEG-PCL, PCL, and PVP-PVAc polymers, respective to the controls (CTRL) (ANOVA, $P < 0.05$ $n = 20$). The electrophysiological (EAG) average responses are presented in alphabetical order. Different letters over the error bars indicate significant differences between the treatments. Uppercase letters correspond to comparisons between the type of nanofiber and their respective control and lowercase letters for comparisons between the different types of nanofibers containing TML.

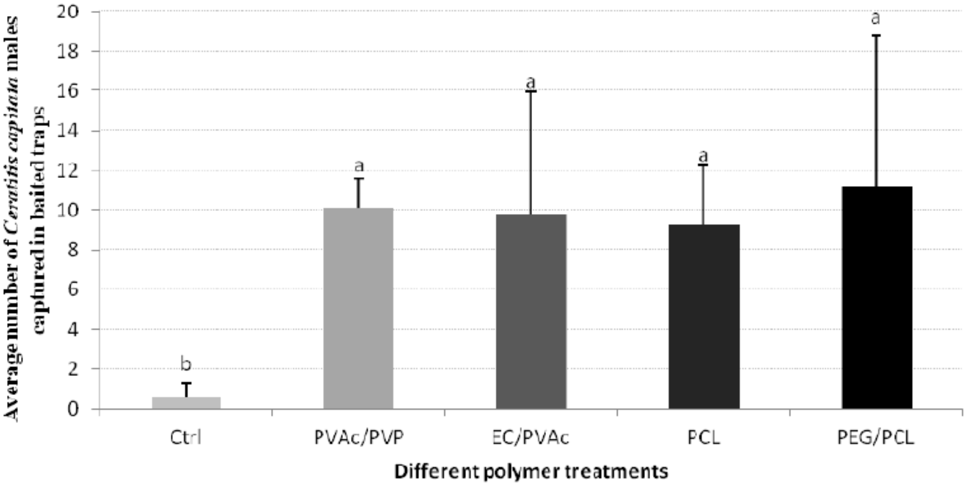

Figure 3: Average number of *Ceratitis capitata* males captured in baited traps with nanofiber adhesive plates made from PVAc-PVP, EC-PVAc, PCL and PEG-PCL polymers containing Trimedlure (TML) attractant with inert adhesive plates as control (CTRL) in bioassays under field cage conditions. The electrophysiological (EAG) average responses are presented in alphabetical order. Different letters on the bars indicate a significant difference in the number of flies captured among the treatments (Kruskal-Wallis H = 20.298, p = 0.0004, n = 10).

to the plates baited with different treatments with the TML than the control (Kruskal-Wallis H = 20,298, p = 0.0004, n = 10) (Figure **3**). All the plates baited with adhesive nanofibers containing TML attracted a similar number of medfly males. Because the nanofibers were effective in releasing the TML and attracted males of *C. capitata* in semi-field conditions, further studies should be conducted to evaluate the potential use of these formulations under field conditions.

The Figures **4** to **7** show photomicrographs from EC, PCL, PEG- PCL and PVAc-PVP nanofibers with and without the addition of trimedlure. It is observed through these figures that there is a modified structure of the fibers after the addition of pheromone. The bead-on-strings shaped nanofibers were becoming smooth with the incorporation of the attractant. This was a general pattern, but the PVAc-PVP nanofibers were predominantly beadless.

The PVAc-PVP nanofibers were smoother than the others and with a larger diameter. The aspect of the fibers is characteristic of the wet-spinning situation.

DISCUSSION

In the field cage conditions bioassay, the inert adhesive plates without incorporated TML did not attract flies, as was expected. There was no significant difference between the numbers of flies caught in the adhesive pads containing the different polymer formulation treatments. All the nanofibers containing TML were attractive, apart from the control (inert plate).

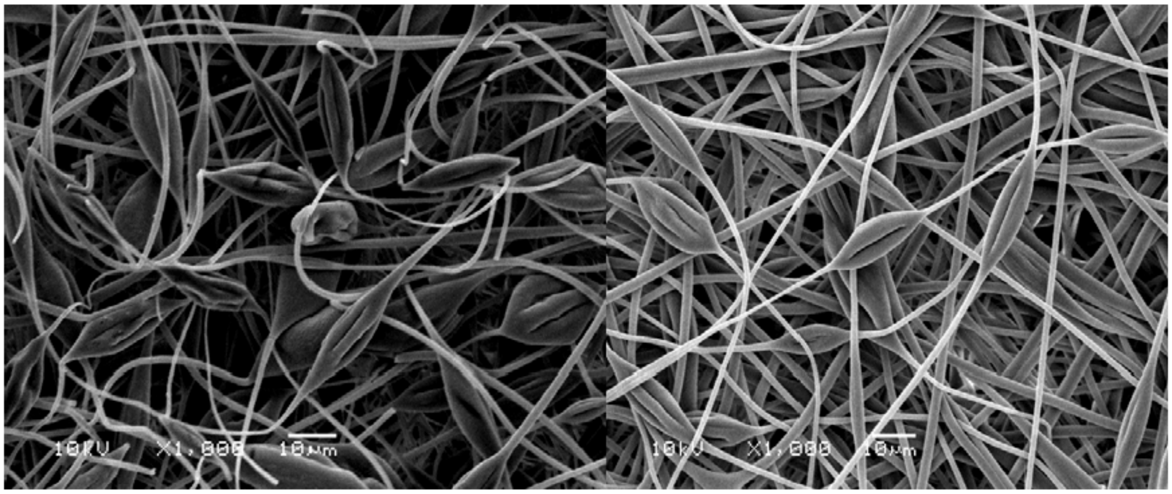

Figure 4: EC nanofibers without (left) and with (right) the addition of pheromone.

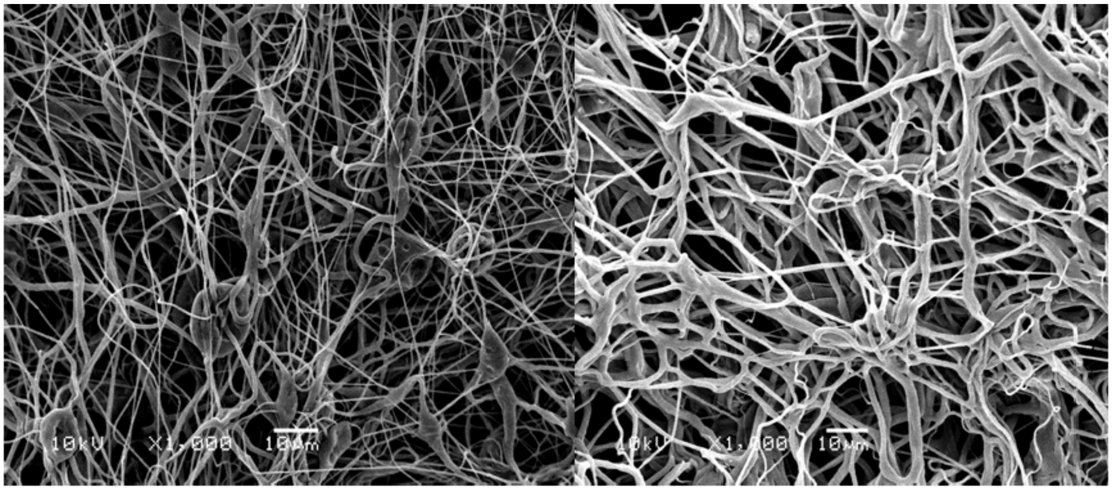

Figure 5: PCL nanofibers without (left) and with (right) the addition of pheromone.

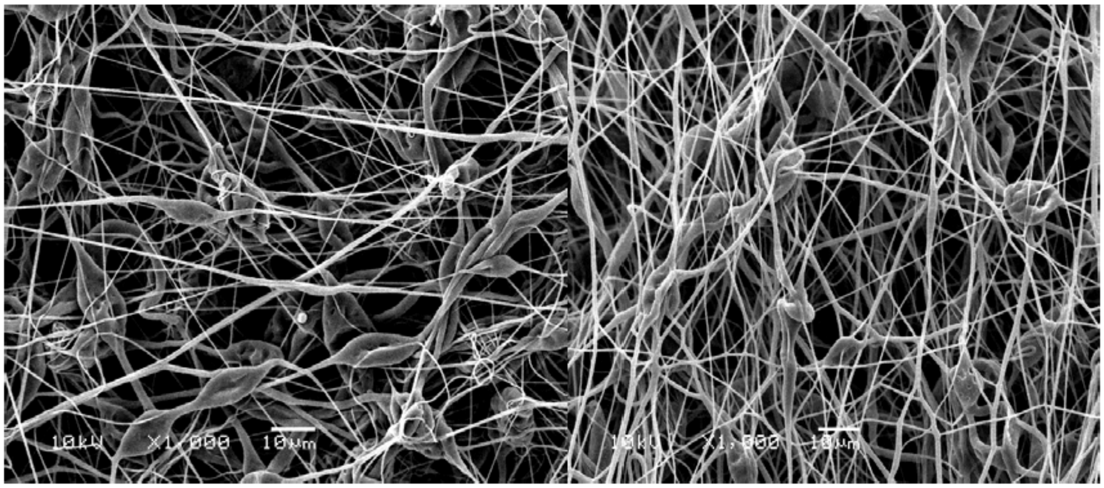

Figure 6: PEG- PCL nanofibers without (left) and with (right) the addition of pheromone.

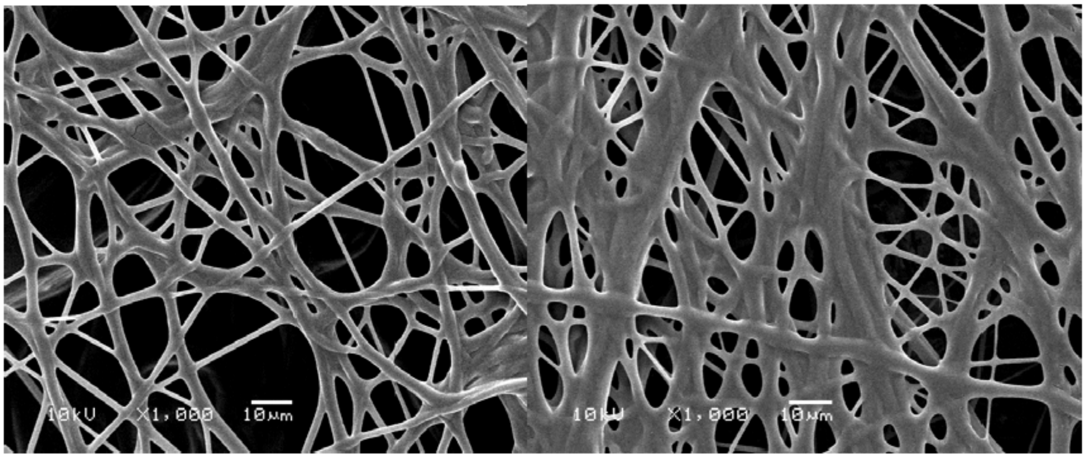

Figure 7: PVAc-PVP nanofibers without (left) and with (right) the addition of pheromone.

The nanofibers were produced by solution or emulsion electrospinning containing TML as a medfly attractant with different polymers and their blends. The polymer selection, the solvent mix used and the oil phase for matching the active ingredient are as important as the agronomical tests employed. The importance of this is further highlighted because the electrospinning technique is an innovative method which is still not well understood. Some of the physicochemical aspects related to the matrix development are discussed, based on the studies made by this present group and others.

Polymer Selection

The common polymers used for pheromone dispersion in fields, such as elastomers and polyethylene, cannot be used for nanofiber or microparticle production. Because of their low dielectric constant and high melting point, they are not the first choice for electro-hydrodynamic techniques, such as electrospinning or electrospraying. For this reason, polymers that are commonly used for controlled release of drugs and that can be easily spinnable or sprayable were chosen. They also served for controlled release. In order to develop innovative pheromone dispersing agents, it is necessary to understand the basic aspects of the novel polymer controlled release matrix.

All solutions formulated with different polymers and TML are chemically compatible so that the flies respond to EAG in the same way as the behaviour bioassays. Cellulose based polymers, like cellulose esters and ethers, are renewable source polymers made from cellulose, which are commonly used for synthetic film making, especially for electrospinning [5]. This study was a pioneer in the use of nanofibers as pheromone dispersers. Besides cellulose derivatives, aliphatic polyesters have been employed as slow-release agents in agriculture for a long time [13]. They degrade into natural materials which are harmless to the environment while slowly releasing the encapsulated herbicides or pesticides. Xu and colleagues (2008) encapsulated doxorubicin hydrochloride in polyester copolymers and Liao and colleagues (2008) encapsulated rhodamine B in poly(lactic-co-glycolic acid) copolymers, using the emulsion method [10,14]. Diclofenac molecules were encapsulated into poly (ε-caprolactone) electrospun fibers for delivery protection [15].

Amphiphilic synthetic polymers are commonly used in tablet and transdermal drug delivery because of the controlled release of the active ingredients [12]. The ester groups present in PVAc and PCL polymer blends were selected because they are environmentally friendly and both are commonly used for the electrospinning technique. The other polymers of the blend were selected for improving the hydrophilic character of the blend, making them amphiphilic and more compatible with the pheromone.

Solvent Selection

Physicochemical parameters, such as viscosity, boiling point and dielectric constant, among others, are fundamental for polymeric solution development for use in making electrospinning and electrospraying fibers or particles. The solvent used in preparing polymer solutions has a significant influence on their spinnability or sprayability capabilities and is fundamental to obtain nanofibers or micro/ nanoparticles with desired properties [16-18]. Solvents should have certain properties, such as good volatility, vapour pressure, high dielectric constant and low boiling point. Thus, for successful electrospinning or electrospraying, the selection of an appropriate solvent system is indispensable. Liu and Hsieh (2002) studied the effect of solvents on spinnability for the formation of smooth nanofibers [19]. Organic solvents were used for each polymer or polymer blend, which were able to dissolve the polymers, having an appropriate viscosity to permit entanglement of the forming fibers, sufficient dielectric constant to direct the fibers or sprays to the collector and high vapour pressure with low boiling point in order to allow the fibers and particles to reach the collector in a dry state.

Effect of Polymer Concentration

When above the critical polymer concentration (c*), 4% in the case of CA, the polymer chains can overlap but do not entangle each other, leading to electrospraying. At a high concentration, above the entanglement concentration (c_e), overlapping of the polymer chains favours entanglement, which gives rise to a much stronger interaction and so leads to smooth fibers [17]. That is the reason for the use of polymers with a concentration range between 5% and 15%.

By the examination of the SEM photomicrographs (Figures **4** to **7**), it was observed through these figures that the bead-on-strings shaped nanofibers (left side) were becoming smoother with the incorporation of the TML attractant (right side of the micrograph). This could have appeared because of the increased conductivity of the solution due to the attractant incorporated in the fibers. The reduction of the beads and fiber diameter are two morphological characteristics that result from the increased polarity of the electrospinning solution or increase of the polymer concentration. The PVAc-PVP nanofibers were smoother and beadless. The aspect of the fibers is characteristic of the wet-spinning case, a situation that occurs when the solvent does not evaporate totally before reaching the fiber collector. This is probably due to the higher boiling point of the solvent mix employed in this case.

CONCLUSION

The nanofiber matrixes prepared by the electrospinning technique can be used for the manufacture of controlled release dispensers of active ingredients, such as insect pheromones, which can be an important tool for the management of insect species that are of interest to the farming community, such as Medfly *Ceratitis capitata*.

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