RESEARCH ARTICLE

Efecto antibacteriano *in vitro* de exudados foliares de tabaco contra dos bacterias fitopatógenas

In vitro antibacterial effect of tobacco leaf exudates against two bacterial plant pathogens

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Resumen

Los productos naturales son una alternativa para el control de microorganismos que ocasionan enfermedades en los cultivos. Este trabajo tuvo como objetivo evaluar diferentes solventes para la obtención de extractos crudos a partir de exudados foliares de líneas de tabaco, y el efecto *in vitro* de estos extractos contra dos bacterias fitopatógenas: *Xanthomonas campestris (Xc)* y *Pectobacterium carotovorum (Pc)*. Se evaluaron solventes con polaridades entre 3.1 y 6.2 (diclorometano, n-butanol, acetato de etilo, metanol y etanol 90 %). El etanol 90 % se seleccionó como mejor solvente y como sustituto del diclorometano por su mayor rendimiento. Los extractos etanólicos crudos se obtuvieron a partir de exudados foliares de diez líneas de tabaco seleccionadas. La diversidad de la composición química de los extractos etanólicos se reveló por cromatografía en capa delgada. La actividad antibacteriana se evaluó por el método de difusión en agar con discos de papel de filtro y la medición del diámetro del halo de inhibición. Se observó inhibición para todos los extractos contra *Xc* destacándose los correspondientes a las líneas Nic 1061 "TI 1738" y Nic 1016 "Incekara" hasta 5 µg de extracto crudo seco /disco, con un mayor rendimiento para la línea Nic 1061. El extracto de la línea Nic 1015 fue el único con actividad contra *Pc* hasta 5 µg de extracto crudo seco por disco. Estos resultados sugieren un uso potencial de los extractos crudos de las líneas Nic 1061 y Nic 1015 "TI 1341" como un agente efectivo para la protección de cultivos contra estas bacterias.

Palabras clave: fitopatógenos, *Nicotiana tabacum,* protección de cultivos, solventes, superficie foliar.

Abstract

Natural products are an alternative to control microorganisms that cause diseases in crops. This work aimed to evaluate different solvents for obtaining crude extracts from tobacco leaf exudates and to determine *in vitro* effect of these extracts against two phytopathogenic bacteria: *Xanthomonas campestris (Xc)* and *Pectobacterium carotovorum (Pc)*. Crude extracts from ten tobacco lines using solvents with polarities between 3.1 and 6.2 (dichloromethane, n-butanol, ethyl acetate, methanol and ethanol 90%) were obtained. Ethanol 90% was selected as the best solvent for obtaining extracts from tobacco leaf exudates and as a substitute of dichloromethane due to the best yield. The chemical composition diversity of the ethanolic extracts was revealed by thin-layer chromatography. The antibacterial activity was evaluated by agar disk diffusion method recording the inhibition zones. Growth inhibition was observed for all extracts against *Xc*, and the better activity corresponded to the lines Nic 1061"TI 1738" and Nic 1016 "Incekara" until a minimal amount of 5 µg/ disc, with higher yield in case of the line Nic1061 . Only the extract of the line Nic 1015 was able to inhibit the growth of *Pc* until a minimal inhibitory concentration of 5 µg/disc. These results suggest a potential use of crude extracts from lines Nic 1061 and Nic 1015 "TI 1341" as an effective agent for the crop protection against *Xc* and *Pc* respectively.

Key words: phytopathogens, *Nicotiana tabacum*, crop protection, solvents, leaf surface.

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Introduction

Plants are a source of food for the animal kingdom and they are affected by pests and diseases that cause severe annual losses. Control of these is generally based on the use of synthetic chemical products, many of which have produced problems of environmental imbalance, human health and the appearance of more aggressive and resistant diseases and pests. There is reference to a high incidence of disease and diverse medical profiles from intoxication in the population, as well as elevated levels of pesticides detected in harvests and people (Martínez *et al*. 2010). The irrational use of fungicides contaminates water and soil. In light of which, the production companies of these compounds are currently obliged to synthesize innocuous and effective ones. This fact has created a discrepancy between the farmers' need to protect their crops from the diseases, and the increasingly greater demand of the markets to provide healthier food (Lara and Landero, 2012). Consequently, other alternatives are sought, which include the use of botanical derivatives for the control of pests and diseases. Several plant species are raw materials for the production of commercial insecticides (Celis *et al*. 2008). Therefore, multiple studies are currently conducted to isolate and identify the secondary metabolites that present defensive activity with the aim to extract them from the plants and use them in disease control (Lara and Landero, 2012).

The selected plants that contain secondary metabolites capable of being used as natural pesticides must be easy to cultivate and have secondary metabolites with powerful activity, high chemical stability and optimum production (Cutler *et al*. 1986). Tobacco is a short-cycle plant considered to be a model of the *Solanaceae* family and very well known for having toxic compounds as defense mechanisms, which makes it attractive for these purposes. The surface of the tobacco leaf is covered in trichomes, most of which are glandular (Akers *et al*. 1978; Wagner, 1991). Glandular trichomes are involved in the secretion of compounds, mainly diterpenes and esters of sugars, followed by waxes and a smaller number of volatile compounds and various others (Severson *et al*. 1984, Wagner, 1999; Tissier, 2012). It is believed that when a large number of these compounds is found on the leaf surface, they are essentially involved in defense against insects and other pathogens (Reuveni *et al*.1987; Wang *et al*. 2004; Shepherd *et al*. 2005; Amme *et al*. 2005; Seo *et al*. 2012).

The isolation of these compounds from the leaf surface using increasingly more efficient and simpler techniques allows these products to be obtained with a greater degree of purity and less cellular contamination due to the advantage that these metabolites have of reproducing in external leaf structures and their secretion on the leaf surface. Several organic solvents have been used for these purposes (Bailey *et al*. 1975; Chang and Grunwald, 1980; Ohnishi *et al*. 1983; Reuveni *et al*. 1987; Lin and Wagner, 1994; Eklund *et al*. 1998; Jackson *et al* 1998; Wang *et al*. 2004), out of which, dichloromethane has been the most used (Severson *et al*. 1984; Wagner *et al*. 2004). Dichloromethane is one of the most used solvents for the extraction of a wide variety of metabolites because it is highly efficient due to its high power of dissolution, excellent recycling property, high stability and elevated purity (Rossberg *et al*. 2006). However, its use presents potential health risks including vertigo, drowsiness and loss of consciousness from inhalation. Other dangers in the use of dichloromethane are its violent reaction with bases and strong oxidants, causing risk of fire and explosion, and its reaction with some forms of plastic, rubber and coverings (ILO, 2005). In recent years, all of this has resulted in a trend to avoid its use and the issuance of new regulations in many countries (ILO, 2005; USDHHS, 2000; SCOEL recommendations 2009; Commission Regulation (EU), 2010), which forces us to look for alternative sources of replacement, because the research focuses on other alternative uses of tobacco to obtain natural products (Wagner *et al*. 2004).

Xanthomonas campestris and *Pectobacterium carotovorum* (also known as *Erwinia carotovora*) are two phytopathogenic bacteria from the soil with an ample range of hosts that include highly important agricultural crops including tomato, potato, cotton, carrot, cucurbit, opinion and capsicum. Therefore, these bacteria belong to the list of the ten most scientifically and economically important bacteria (Mansfield *et al*. 2012) and to date, fully effective protection against them has not been developed.

The overall objective of this work is to obtain crude extracts from the leaf exudates of different lines of tobacco for their later use in the control of pests and diseases in crops of agricultural importance. Its specific objectives are to establish the solvent capable of replacing dichloromethane when obtaining crude tobacco extracts active against pathogenic bacteria and to establish the *in vitro* antibacterial activity of these crude extracts obtained from several contrasting lines of tobacco against these two plant pathogen bacterial as a first step toward the selection of candidate lines of tobacco to obtain natural products for the control of bacterial diseases.

Materials and Methods

Bacterial strains: The pathogenic strains *Xanthomonas campestris,* isolated from tomato, and *Pectobacterium carotovorum,* isolated from potato, were provided by the Instituto de Sanidad Vegetal, Habana, Cuba, and were preserved in nutritive agar containing 4% glycerol at 4°C until their use.

Vegetable Material: The ten tobacco accessions were planted in seedbeds and floating trays (Cristanini, 1995) in the Estación de Tabaco de Cabaiguán, Sancti

Spiritus, Cuba. The following lines were provided by the Germoplasm Bank of IPK-Gatersleben, Germany: Nic 1003 ("TI 66"), Nic 1006 ("TI 193"), Nic 1015 ("TI 1341"), Nic 1016 ("Incekara"), Nic 1017 ("Red Russian"), Nic 1019 ("TI 998"), Nic 1061 ("TI 1738") and SNN (Samsung). The BHmN and CE (Corojo Especial) lines were provided by the Centro de Ingeniería Genética y Biotecnología, Habana, Cuba.

At 45 days, the plants were separated into individual plants and transplanted to plastic bags for a total of ten plants per line. Seventy days after their transplantation to bags, indicators were measured such as the number of leaves, plant height and fresh leaf mass. The data analysis was carried out with the Statistical Package for Social Sciences (SPSS) (Version 15 para Windows, SPSS Inc.). Parametric tests were conducted (ANOVA of one factor and Tukey's test) and the differences between the means were analyzed for $p \leq 0.05$.

Experiment 1: Establishing the Solvent for Obtaining the Crude Extracts of Tobacco Leaf Exudates

With the aim to select the best solvent to obtain the crude extracts of tobacco leaf exudates, five solvents were evaluated with polarities between three and six: dichloromethane (3.1), n-butanol (3.9), ethyl acetate (4.4), methanol (5.1) and 90% ethanol (6.2).

The five to six youngest leaves of two plants of the Nic 1061 line were used for each treatment, which corresponded to a fresh mass of approximately 60 g. The plants had an average height of 80 cm. The leaves were submerged in 500 ml of the solvent four times for seven seconds, they were filtered through paper, concentrated by rotary evaporation to a volume of 50 ml and kept at -20 °C until their later evaluation. A yield expressed in milligrams of dry mass of the extract / grams of fresh leaf mass was established.

To establish the presence of secondary metabolites in the extracts of leaf exudates obtained from different solvents, they were run in chromatoplates of silica gel (DC-Cards SI F 20 x 20 cm, Riedel de la Haën). The Petri dish was activated for 10 minutes at 60 °C and the application was carried out with ten drops per sample. It was used as an eluent system: benzene: ethyl acetate (86:14). After the running of the chromatoplates, they were dried for two to three minutes at 60 °C. The development was carried out with a phosphomolybdic acid solution (20 g of phosphomolybdic acid in 100 ml of absolute ethanol.

Experiment 2: Obtaining Crude Extracts from Leaf Exudates of Lines of Tobacco

Three plants were taken from the ten lines planted and the number of leaves, plant height, fresh leaf mass and dry leaf mass were measured to establish the dry mass / fresh mass rate.

Extraction from the leaves of three plants was carried out in the same way as in the previous experiment using 90% ethanol as a solvent. The concentration of the extracts expressed in milligrams of dry mass·ml^{-1} of solvent (data not shown) and the yield expressed in milligrams of dry extract·g^{-1} of fresh leaf mass were established. Thin-layer chromatography was also carried out, as explained in the previous experiment.

Experiment 3: Establishment of the Antimicrobial Activity of the Tobacco Extracts Obtained from Phytopathogenic Bacteria

The trial of antibacterial activity was conducted by the agar diffusion method using filter-paper discs (Valgas *et al.* 2007). The Petri dishes were inoculated with each bacterium (*Xanthomonas campestris and Pectobacterium carotovorum*) to OD 625 nm of 0.15 and 0.17, respectively. An aliquot of 100 µL of bacterial solution was extended above the Luria Bertani (LB) medium.

The dry extracts were dissolved with dimethyl sulfoxide (DMSO) at 5 and 25 µg·µL^{-1}. With the aim to establish the antimicrobial activity of the crude extracts obtained from the different solvents and those obtained from the different lines using the selected solvent (Experiments 1 and 2), 20 µL of the concentration 25 µg·µL^{-1} was applied to sterile filter paper discs measuring 0.7 cm in diameter.

With the aim to establish the different concentrations of the extracts, 1, 5, 10, 15 and 20 µL (5, 25, 50, 75 and 100 µg, respectively) of the 5 µg·µL^{-1} concentration and 8, 12, 16 and 20 µL (200, 300, 400 and 500 µg, respectively) of the 25 µg·µL^{-1} concentration were applied to sterile filter paper discs measuring 0.7 cm in diameter.

The discs with the extracts were dried and placed at a distance of 1.5 - 2.0 cm between them in the Petri dishes, previously inoculated with each bacterium. The kanamycin solution at 50 µg·µL^{-1} (10 µL) was used as a positive control in the *in vitro* trial as the bacterium susceptible to this antibiotic. Dimethyl sulfoxide was used as a negative control or solvent control (20 µL) for all the cases, because this was the solvent used to dissolve the dry extracts.

The Petri dishes were incubated for 24 hours at 37 °C and the diameter of the halo of inhibition was measured, including the diameter of the disc. Each treatment was carried out three times and the experiment twice.

Results and Discussion

Seventy days after the transplant to bags, the lines with the greatest height and number of leaves were Nic 1016, SNN and Nic 1061 (Table 1). These two indicators tend to be very closely related because as the plant grows in height it grows a greater number of leaves.

The plants with the greatest height were found in the mentioned lines, but without difference in the CE Line, covering values between approximately 60 and 68 cm, which corresponds to a small height for the age of the plants, but a normal height for the conditions of bags in a greenhouse. The plants with a medium height presented values between approximately 40 and 55 cm. The line with the smallest height was BHmN without difference to Nic 1015, which showed around 35 cm (Table 1).

Three groups are defined for the number of leaves. These are the first group comprised of the Nic 1016, SNN and Nic 1061 Lines with the greatest values from 18 to 19 leaves per plant, the second group in which the Nic 1003 Line is found with 16 leaves and the third group having the greatest number of lines with values between 12 and 14 leaves per plant (Table 1).

The total fresh mass of leaves of three plants per line varied between approximately 50 g and 450 g, which is influenced by the number of leaves per plant, although lines with a lower number of leaves showed high values of fresh mass (BHmN, Nic 1019 and CE) (Table 1). The dry leaf mass / fresh leaf mass rate allows us to analyze which lines present a greater production of metabolites in the leaves. In this aspect, the Nic 1016, SNN and Nic 1006 Lines stand out with values of around 0.7 and the rest of the lines present intermediate values between 0.4 and 0.5, with the exception of the BHmN Line, which shows the lowest rate with a value of 0.3. Although this aspect is important, it is not a determining factor in our case, because we focus on the compounds of the leaf surface that are included but less represented in the total metabolites of the leaf.

The number of leaves per plant could be a criterion to take into account for the purpose of obtaining extracts from this vegetable organ. Therefore, for the experiment of different solvents, we selected the Nic 1061 Line with the greatest number of leaves, height and intermediate values of fresh mass, and dry mass / fresh mass rate.

Table 1. Morphological indicators of ten lines of tobacco 70 days after transplant to bags.

Lines	Total Fresh Leaf Mass (g)	Dry Leaf Mass / Fresh Leaf Mass Rate	Plant Height (cm)	Number of Leaves per Plant
Nic 1016	447.30 a	0.64 b	63.88 a	19 a
BHmN	192.10 b	0.31 f	32.75 f	12 c
Nic 1019	189.40 b	0.43 de	48.13 cd	13 c
CE	145.10 c	0.52 c	59.75 ab	13 c
SNN	126.00 d	0.70 a	68.25 a	18 a
Nic 1061	102.00 e	0.39 e	65.50 a	18 a
Nic 1017	93.70 ef	0.38 e	53.63 bc	14 c
Nic 1006	86.70 f	0.64 b	45.13 cd	13 c
Nic 1003	67.20 g	0.47 cd	41.50 de	16 b
Nic 1015	48.80 h	0.52 c	34.88 ef	13 c

Means with different letters represent significant differences between the lines of tobacco for each indicator (ANOVA of one factor and *Tukey's* test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=3).

Experiment 1: Establishing the Solvent for Obtaining the Crude Extracts of Tobacco Leaf Exudates

Greater yields were obtained in this experiment with the use of 90% ethanol, followed by dichloromethane and finally the solvents methanol, ethyl acetate and n-butanol without differences between them (Figure 1 A). The increase in the polarity of 90% ethanol, also taking into account the 10% water which is present, could have contributed to the extraction of other more polar compounds that were not extracted with the other solvents, and these increase the mass of the dry extract and obviously the yield. Therefore, this solvent may have the capacity to extract polar and less polar compounds.

The thin-layer chromatography of the extracts developed effective and similar extraction of the majority compounds in all the cases (Figure 1 B). All of the solvents evaluated were capable of extracting the majority compounds without great differences in the chromatographic pattern. In all of the extracts obtained with different solvents, Compound A is developed close to the point of application and Compound B close to the front of the running gel, with a greater intensity of Compound A for the ethanolic extract, followed by the methanolic extract, and less for the butanolic extract (Figure 1 B).

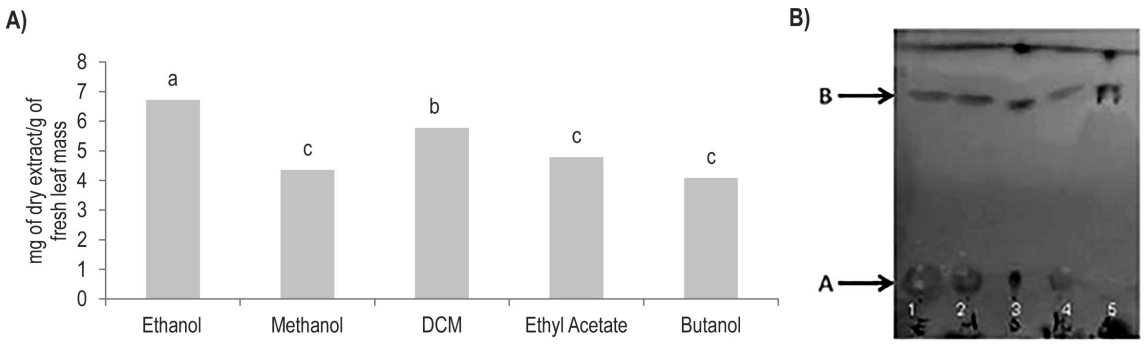

Figure 1 A. Yield of the extraction in milligrams of dry extract $\cdot g^{-1}$ from fresh leaf mass of the exudates obtained in 500 ml of the different solvents: 90% ethanol, methanol, DCM (dichloromethane), ethyl acetate and n-butanol from the Nic 1061 Line. B. Thin-layer chromatography for the separation of the crude extracts obtained from leaf exudates of the Nic 1061 Line with different solvents. 1. Extract with 90% ethanol. 2. Extract with methanol 3. Extract with dichloromethane 4. Extract with Ethyl acetate 5. Extract with butanol. Means with different letters represent significant differences between solvents (ANOVA of one factor and Tukey's test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=6).

Due to the fact that dichloromethane has become a factor of reference for washing the surfaces of tobacco leaves, there is little data published in the last decade regarding the extraction efficiency of other solvents for these purposes (Whitfield, 2004). There is a greater tendency to use tobacco in the production and obtaining of natural products, which is why the development of scalable extraction methods is a determining factor.

With the aim to develop methods for the production and extraction of proteins from the surface of transgenic tobacco leaves, Whitfield (2004) also evaluated different solvents and the number of washes. The studies showed that a greater number of washes of the evaluated solvents (three washes) can, in some cases, achieve yields comparable with those achieved with dichloromethane. They also demonstrated that the individual extraction of each compound also depended on the number of washes, but in all cases, 100% of the amount of each compound was achieved after the three washes. In general, the best results were obtained with n-propanol, methanol and ethanol.

In this experiment, the four washes carried out for all the solvents must allow greater extraction of metabolites in general of 90% ethanol compared to dichloromethane and the rest of the solvents in equal conditions. Therefore, the intensity of the A and B Compounds seems to correspond to the maximum for each compound, which establishes that n-butanol is not capable of extracting equal amounts of the B Compound in these equal conditions if we compare it to the rest of the solvents evaluated. However, a study of the chemical composition of all the extracts is required to reach this conclusion.

The effect of deterioration that dichloromethane causes in the leaves is also known. This experiment observed some damages not evaluated in the leaves treated with dichloromethane and that the leaves treated with 90% ethanol were in a better condition. This has been proven by Whitfield (2004), who observed a small effect on the conditions of the leaves when solvents such as ethanol and methanol were used, a medium effect on the leaves treated with isopropanol and n-propanol and a large effect on the leaves treated with dichloromethane. Therefore, this is an aspect to take into account in the selection of the solvent, if the intention is to integrate the step of extraction of compounds from the surface in bioprocesses or chemical analysis of the inside of the leaf.

Taking into account the yields in the dry extracts (greater in 90% ethanol) and the similarity in the chromatographic pattern of the extracts of dichloromethane and ethanol, and even when the methanol and ethyl acetate solvents present similar patterns, we propose the use of 90% ethanol when obtaining leaf exudates from the different lines, because it is more efficient than dichloromethane and the rest of the solvents. This is in addition to the other considerations, for example that 90% ethanol is more innocuous, safer, less aggressive, easier to handler, has an easier concentration and recovery and is more readily available and cheaper.

The 90% ethanol solvent was selected to obtain crude extracts for the next experiments. However, the selection of the most adequate solvent always depends on the compounds to isolate and their activity. Therefore, it is possible that 100% effectiveness is not achieved due to the great diversity of chemical structures present in the surface of the tobacco leaf between the different genotypes, but at least a good representation of the majority compounds is achieved. It is necessary to prove the activity of the isolated compounds after extraction.

Experiment 2: Obtaining Crude Extracts from Leaf Exudates of Lines of Tobacco

Tobacco leaf exudates were obtained from ten lines of tobacco using 90% ethanol as a solvent. The crude extracts up to 50 ml showed different intensity in coloring (data not shown), which corresponded to the different concentrations in milligrams of dry extract·ml of 90% ethanol (data not shown). The lines presented a different yield between them (Figure 2 A).

The greatest yield was achieved from the Nic 1006 Line with values of 8 mg of dry extract· mg^{-1} of fresh leaf mass, followed by the Nic 1003 Line, and then the Nic 1019 and Nic 1015 Lines. The latter without a difference to the Nic 1061 Line, covering values between 4 and 7 mg of dry extract·mg^{-1} of fresh leaf mass. The lowest yield was obtained from the Nic 1016 Line (Figure 2 A), which despite having a large fresh leaf mass, height and number of leaves per plant (Table 1), great amounts of components were not extracted from the leaf surface. This demonstrates that the morphological indicators are not determining factors in the selection of the candidate lines; the chemical composition of the lines is more related to the genetic factor. However, once the genetic capacity for synthesis of the compounds of interest is detected, knowledge of the morphological aspects is important at the time of establishing a production process.

The thin-layer chromatography of the extracts in these conditions developed a diversity in chemical composition produced by the line of tobacco, as well as the concentration of the extracts and the majority compounds in all the cases (Figure 2 B). In the majority of the extracts, a Compound A is developed close to the point of application that does not appear in Extracts 3 and 9 (Figure 2 B), and a Compound B is developed close to the front of the running gel that does not appear in Extracts 2 and 8 (Figure 2 B). Compounds C and D are shown in Extracts 4 and 6 (Figure 2B). The presence of a Compound E is observed in Lines 1, 4 and 5, and of Compound F in Lines 1, 4, 5 and 6 (Figure 2 B). Compound G, which is closer to the front of the running gel, is shown only in Extracts 5 and 6 (Figure 2 B).

This system permits the separation of different components of a sample and the demonstration of the chemical diversity that the ethanolic extracts obtained, because of the great chemical diversity characteristic of the *Solanaceae* family in general and the diversity of genotypes (Wollenweber *et al.* 2005; Cui *et al.* 2011; Tissier, 2012, Sallaud *et al.* 2012), which makes a wide range of potential applications of these extracts possible. Other chromatography methods are required for the quantification and identification of the compounds present.

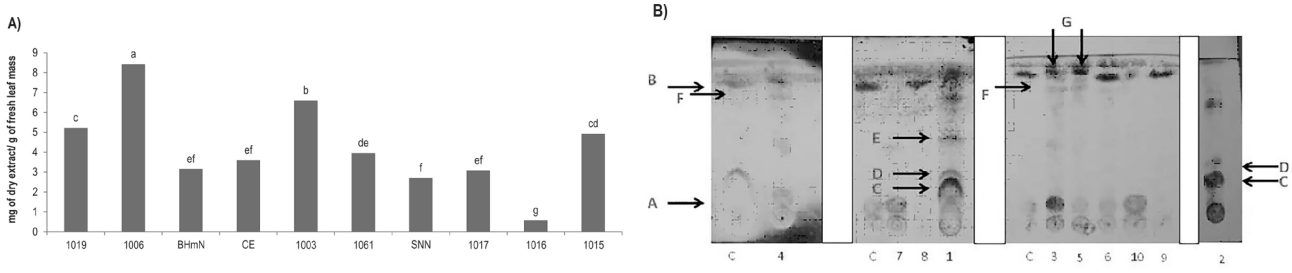

Figure 2 A. Yield of the extraction in milligrams of dry extract·g^{-1} from fresh leaf mass of the exudates obtained in 500 ml of 90% ethanol from the ten lines. B. Thin-layer chromatography for the separation of the crude extracts. The ethanolic extract obtained from Line 1061 in Experiment 1 of different solvents was used as a control. 1: CE, 2: SNN, 3: Nic 1017, 4: Nic 1019, 5: BHmN, 6: Nic 10103, 7: Nic 1061, 8: Nic 1015, 9: Nic 1016, 10: Nic 1006. Different compounds developed are indicated with the letters A-G. Means with different letters represent significant differences between lines of tobacco (ANOVA of one factor and Tukey's test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=6).

Although the secondary metabolites are those most frequently associated with resistance to diseases, defensive proteins called phylloplanins related to pathogenesis form part of the surface of tobacco leaves and are considered products of glandular trichomes, conferring resistance to the pathogens (Sheperd *et al.* 2005). In this case, with the use of medium polarity solvents, the extraction of the majority secondary metabolites from the leaf surface such as diterpenes and esters of sugars is promoted. Due to the fact that the phylloplanins are polar compounds, only aqueous extractions would allow phylloplanins to be obtained in the extracts. Therefore, they are not expected to be present in the extracts obtained (Sheperd *et al.* 2005).

All of the extracts are selected for the evaluation of *in vitro* antibacterial activity against the pathogenic bacteria *Xanthomonas campestris* and *Pectobacterium carotovorum*.

Experiment 3: Establishment of the Antimicrobial Activity of the Tobacco Extracts Obtained from Phytopathogenic Bacteria

When establishing the antibacterial activity of extracts obtained for the comparison of different solvents against *Xc* and *Pc*, the Nic 1061 Line showed its capacity to inhibit the growth of *Xc* with all the extracts (Table 2). An analysis of halos of inhibition did not develop significant differences between the extracts, which caused an inhibition of approximately 2.0 cm in diameter. This shows two results. The extracts of the Nic 1061 Line inhibit the growth of *Xc* and the evaluated solvents are capable of extracting the active compound or the active compounds in similar quantities.

The kanamycin used as a positive control caused an inhibition of 3.0 cm in diameter, significantly different to that of the extracts for the case of *Xc*. However, in the trial against *Pc*, growth inhibition was not shown by the extracts and there was a lower inhibition caused by kanamycin of approximately 2.0 cm in diameter. The use of kanamycin only allows us to achieve a growth inhibition indicator in bacteria susceptible to it with *in vitro* trials, but this antibiotic is not used *in vivo*, and it is not possible to carry out a real comparison of its effect with the effect that the crude extracts could cause, which are comprised of a mixture of compounds. Other authors have used kanamycin and other antibiotics on *in vitro* trials of antibiograms (Pino *et al.* 2012; Owoseni and Sangoyomi, 2014). In future experiments, kanamycin could be used in very low concentrations to prevent its influence on the discs evaluated in a reduced space of the Petri dishes. Also, a smaller number of discs could be evaluated per Petri dish to prevent overlapping of halos if the products applied show a great inhibition of bacterial growth.

Table 2. *In vitro* antibacterial effect of extracts from tobacco leaf exudates *(N. tabacum L.)*, Nic 1061 Line, obtained from the use of different solvents against the phytopathogenic bacteria: *Xanthomonas campestris (Xc)* and *Pectobacterium carotovorum (Pc).* The inhibition zone is established by the diameter expressed in centimeters (cm) and includes the diameter of the disc (0.7 cm). K: kanamycin as a positive control (500 µg).

	Solvents Used and Diameters of Halos of Inhibition of Bacterial Growth (cm), Nic 1061 Line					
Bacteria	**90% Ethanol**	**Dichloromethane**	**n-Butanol**	**Methanol**	**Ethyl acetate**	**K**
Xc	2.1 b	2.2 b	1.7 b	2.1 b	2.1 b	3.0 a
Pc	NI	NI	NI	NI	NI	2.5 a

Means with different letters represent significant differences between solvents and kanamycin for each independent bacterium. (ANOVA of one factor and Tukey's test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=6).

The dimethyl sulfoxide used to dissolve the dry extracts did not show growth inhibition when it was evaluated as a negative control. This demonstrates that this solvent did not interfere in the results obtained and demonstrates its effective use for these experiments.

Geethaa *et al.* (2013) showed that dimethyl sulfoxide was the safest solvent in a comparison between solvents to detect phytotoxic effects and the presence of bioactive compounds.

The similarity found in the inhibition trials for the extracts obtained from the use of different solvents supports the idea of replacing dichloromethane with 90% ethanol for this purpose in particular, because this corroborates that the same compounds have been extracted which have antibacterial properties against *Xc*. This line showed activities for just one bacterium, which indicates that all the extracts must be evaluated against the two bacteria and suggests the existence of different active compounds for the interaction with different bacteria.

When establishing the bacterial activity of extracts obtained for the comparison of lines of tobacco against *Xc* and *Pc,* it was observed that all of the crude extracts evaluated showed a smaller halo of inhibition compared to kanamycin in the trials of both bacteria (Figure 3). For the case of *Xc*, the best extracts were obtained from the Nic 1019 (4), BHmN (5), Nic 1061 (7) and Nic 1016 (9) Lines, while in the case of *Pc*, only the extract of the Nic 1015 (8) Line showed an inhibitory effect (Table 3) (Figure 3). Therefore, it is possible to obtain active extracts against these two bacteria using more common, less toxic and cheaper solvents than dichloromethane.

For the trial of different concentrations lower than 500 µg/disc, the extracts of the BHmN (5), Nic 1061 (7) and Nic 1016 (9) Lines against *X. campestris* and the extract of the Nic 1015 (8) Line against *P.carotovorum* were selected. Extracts 7 and 8 showed inhibition of the *X. campestris* and *P. carotovorum* bacteria, respectively, at the concentration of 5 µg/disc with significant differences in concentrations between 300 and 500 µg/disc for the Nic 1061 Line and between 400 and 500 µg/disc for the Nic 1015 Line (Table 4).

Table 3. *In vitro* antibacterial effect of ethanolic extracts (500 µg) of tobacco leaf exudates (*N. tabacum* L.) (ten lines) against the phytopathogenic bacteria *Xanthomonas campestris (Xc)* and *Pectobacterium carotovorum (Pc).* The inhibition zone is established by the diameter expressed in centimeters (cm) and includes the diameter of the disc (0.7 cm). K: kanamycin as a positive control (500 µg).

	Lines of Tobacco Evaluated and Diameter of Halo of Inhibition of Bacterial Growth (cm)										
Bacteria	**CE**	**SNN**	**Nic 1017**	**Nic 1019**	**BHmN**	**Nic 1003**	**Nic 1061**	**Nic 1015**	**Nic 1016**	**Nic 1006**	**K**
Xc	1.3 c	1.3 c	1.4 c	1.7 b	1.7 b	1.5 c	1.9 b	1.4 c	1.8 b	1.5 c	3.3 a
Pc	NI	NI	NI	NI	NI	NI	NI	1.6 b	NI	NI	2.6 a

Means with different letters represent significant differences between the lines of tobacco and kanamycin for each independent bacterium (ANOVA of one factor and Tukey's test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=6).

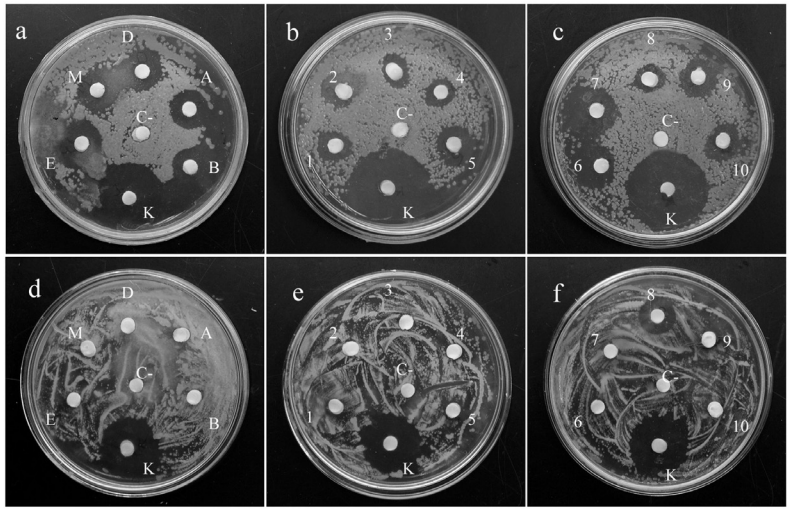

Figure 3. *In vitro* antibacterial effect of crude extracts (500 µg) from tobacco leaf exudates *(N. tabacum L.)* through the method of diffusion in agar with filter paper discs against two phytopathogenic bacteria, *Xanthomonas campestris* (A, B, C) and *Pectobacterium carotovorum* (D, E, F), established by the halo of inhibition of bacterial growth. Extracts obtained with five solvents (E: 90% ethanol, M: methanol, D: dichloromethane, A: ethyl acetate, B: n-butanol), Nic 1061 Line, against *Xanthomonas campestris* (a) and *Pectobacterium carotovorum* (d). Ethanolic extracts obtained from ten lines of tobacco 1: CE, 2: SNN, 3: Nic 1017, 4: Nic 1019, 5: BHmN, 6: Nic 10103, 7: Nic 1061, 8:Nic 1015, 9: Nic 1016, 10: Nic 1006 against *Xanthomonas campestris* (b, c) and *Pectobacterium carotovorum* (e, f). K: kanamycin as a positive control (500 µg). C-: dimethyl sulfoxide as a negative control.

Table 4. *In vitro* antibacterial effect of different concentrations of ethanolic extracts of tobacco leaf exudates *(N. tabacum* L.) of lines selected due to their antibacterial activity against the phytopathogenic bacteria *Xanthomonas campestris (Xc)* and *Pectobacterium carotovorum (Pc).* The inhibition zone is established by the diameter expressed in centimeters (cm) and includes the diameter of the disc (0.7 cm). K: kanamycin as a positive control (500 µg).

Amount of Extract Applied to Each Filter Paper Disc (µg) Diameter of Halo of Inhibition of Bacterial Growth (cm)											
Bacteria	Lines	5	25	50	75	100	200	300	400	500	K
Xc	BHmN	NI	1.0 c	1.1 c	1.4 bc	1.5 bc	1.4 bc	1.6 b	1.6 b	1.6 b	3.0 a
	Nic 1061	0.9 c	0.9 c	1.3 bc	1.4 bc	1.5 bc	1.5 bc	1.8 b	1.8 b	1.8 b	3.1 a
	Nic 1016	NI	0.8 c	1.2 bc	1.3 bc	1.5 b	1.6 b	1.5 b	1.5 b	1.6 b	2.9 a
Pc	Nic 1015	0.8 c	0.9 bc	1 bc	0.9 bc	1 bc	1.0 bc	1.0 bc	1.4 b	1.4 b	1.9 a

Means with different letters represent significant differences between concentrations for each independent line of tobacco (ANOVA of one factor and Tukey's test in a statistical analysis conducted in SPSS for $p \leq 0.05$, n=6).

Shobha and Kale (2008) found active, *in vitro* extracts against pathogenic fungi and bacteria of the soil, which include *Xanthomonas campestris* and *Erwinia carotovora*, but from the preparations obtained from exudates of the *Eudrilus Eugeniae* earthworm.

Similar experiments were conducted by Owoseni and Sangoyomi (2014) in search of efficient solvents to obtain extracts of active plants against the phytopathogenic bacteria *Ralstonia solanacearum,* which affects members of the *Solanaceae* family, and they showed more active *in vitro* extracts from chloroform, followed by methanol and ethanol with diameters of halos of inhibition between 0.8 and 1.5 cm.

Seo *et al.* (2012) identified two natural diterpenes present in tobacco (sclareol and cis–abienol) that inhibited the disease caused by *Ralstonia solanacearum* in tobacco, tomato and Arabidopsis plants after applying the product to the roots without having shown antibacterial activity. This suggests that it is possible that the terpenes present in the crude extracts are involved in the antibacterial response, but according to the mechanism of action, these could serve as defense inducers in the plant even when they have not shown direct inhibition of the bacterial growth.

The selection of candidates to obtain crude extracts that have an inhibitory effect on the phytopathogenic bacteria of interest is based on the first criterion of these ethanolic extracts demonstrating an inhibitory *in vitro* effect on the pathogens. Other criteria to take into account are that the plant in the extraction process presents large yields of metabolites in general, as well as some morphological indicators that could be related to the synthesis of the natural product. However, even when the number of leaves is one of the lowest and the yields are intermediate, according to our results for the control of *P. carotovorum*, the extract obtained from the Nic 1015 Line is selected as a potential candidate, because it was the only one that showed an evident inhibition of the bacterium's growth. In the case of *X. campestris*, four extracts show a similar inhibitory effect. Therefore, this criteria in combination with the results in number of leaves and yields leads to the selection of the Nic 1061 Line with the greatest number of leaves and intermediate yield. However, the Nic 1019, BHmN And Nic 1016 Lines can also be considered as potential candidates for obtaining ethanolic leaf exudates in the control of *X. campestris*. A combination of a single product from these selected extracts could produce a good result in the control of both bacteria.

Conclusions

The 90% ethanol solvent was selected as the best solvent for obtaining crude extracts from tobacco leaf exudates, and crude ethanolic extracts were obtained from tobacco leaf exudates that showed a chemical diversity in terms of concentration, composition and yield, as well as *in vitro* antibacterial activity against the phytopathogenic bacteria evaluated: *X. campestris* and P. *carotovorum,* for some cases. These results suggest the potential use of ethanolic extracts obtained from the Nic 1061 and Nic 1015 Lines as the best candidates against *X. campestris* and *P. carotovorum,* respectively, as an effective agent for the protection of crops, with prior evaluation using *in vivo* conditions in a greenhouse and in the field as an alternative to the use of chemical bactericides for the protection of crops of agricultural interest.

Bibliographic References

Akers, C. P., Weybrew, J. A., y Long, R. C. (1978). Ultrastructure of glandular trichomes of leaves of *Nicotiana tabacum* L. cv Xanthi. *American Journal of Botany,* 65, 282-292.

Amme, S., Rutten, T., Melzer, M., Sonsmann, G., Vissers, J.P.C., Schlesier, B., y Mock, H. P. (2005). A proteome approach defines protective functions of tobacco leaf trichomes. *Proteomics,* 5, 2508-2518.

Bailey, J. A., Burden, R. S., y Vincent, G. G. (1975). Capsidiol: An antifungal compound produced in *Nicotiana tabacum* and *Nicotiana clevelandii* following infection with tobacco necrosis virus. *Phytochemistry,* 14, 597.

Celis, A., Mendoza, C., Pachón, M., Cardona, J., Delgado, W., y Cuca L.E. (2008). Extractos vegetales utilizados como biocontroladores con énfasis en la familia *Piperaceae*. Una revisión. *Agronomia Colombiana,* 26 (1), 97-106.

Chang, S. Y., y Grunwald, C. (1980). Structural organization of tobacco leaf polar cuticular lipids. *Botanical Gazette*, 141(4), 360-365.

Commision Regulation (EU) No 276/2010 of 31 March 2010. Official Journal of the European Union, L 86/11. Consultado el: 02-07-2014.

Cristanini, G. (1995). La producción de posturas en sistemas flotantes (float system)/ hidropónicos. *L' informatore agrario/ suplemento*. 51 (5), 43-53.

Cui, H., Zhang, S., Yang, H., Ji, H., y Wang, X. (2011). Gene expression profile analysis of tobacco leaf trichomes. *BMC plant biology*, 11:76.

Cutler, H. G., Severson, R. F., Cole, P. D., Jackson, D. M., y Johnson, A. W. (1986). Secondary metabolites from higher plants in M.B. Green and P.A. Hedin (eds.). Natural Resistance of Plants to Pests: Roles of Allelochemicals. *American Chemical Society Symposium Series* No. 296, Washington, D.C. 178-196.

Eklund, A. M., Forsblom, I., Berg, J. E., Damberg, C., y Wahlberg, I. (1998). Tobacco chemistry. Four new cyclized cembranoids from tobacco. *Acta Chemica Scandinavica,* 52, 1254- 1262.

Geethaa, S., Thavamany, P. J., Chiew, S. P., y Thong, O. M. (2013). Interference from ordinarily used solvents in the outcomes of *Artemia salina* lethality test. *Journal of advanced pharmaceutical technology & research,* 4 (4), 179-182.

International Labour Organization (ILO). (2005). International Occupational Safety and Health Information Centre (CIS). International Chemical Safety Cards [en línea]. [Geneva, Switzerland]: abril 2000; actualizado abril 2005 [citado julio de 2007]. DICHLOROMETHANE.http://www.ilo.org/public/english/protection/safework/cis/products/icsc/dtasht/index.htm. Consultado 07-07-14.

Jackson, D. M., Chortyk, O. T., Stephenson, M. G., Johnson, A. W., Harlow, C. D., Simmons, A. M., y Sisson, V. A. (1998). Potential of *Nicotiana* species for production of sugar esters. *Tobacco Science,* 42 (1), 1-9.

Lara, F. M., y Landero N. (2012). Químicos vegetales: alternativa contra los agentes patógenos. *Cienc. hombre,* XXV (1). Enero-Abril.

Lin, Y., y Wagner G. J. (1994). Rapid and simple method for estimation of sugar esters. *Journal of Agricultural and Food Chemistry,* 42, 1709-1712.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S., Machado, M., Toth, I., Salmond, G., y Foster, G. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular plant pathology,* 13(6), 614-629.

Martínez, S., Terrazas, E., Álvarez, T., Mamani, O., Vila, J., y Mollinedo, P. (2010). Actividad antifúngica *in vitro* de extractos polares de plantas del género *baccharis* sobre fitopatógenos, *Revista Boliviana de Química,* 27(1), 13-18.

Ohnishi, A., Chinju, M., y Katö, K. (1983). Isolation and characterization of cuticular polyester from the leaf of *Nicotiana tabacum*. *Agricultural and Biological Chemistry*, 47(5), 975-981.

Owoseni, A. A., y Sangoyomi, T. E. (2014). Effect of Solvent Extracts of Some Plants on *Ralstonia solanacearum*. *British Microbiology Research Journal,* 4(1), 89-96.

Pino, O., Sánchez, Y., Rojas, M.M, Abreu, Y., y Correa, T.M. (2012). Composición química y actividad antibacteriana del aceite esencial de *Pimpinella anisum* L. *Revista Protección Vegetal.* 27 (3), 181-187.

Reuveni, M., Tuzun, S., Cole, J. S., Siegel, M. R., Nesmit, W. C., y Kué, J. (1987). Removal of duvatrienediols from the surface of tobacco leaves increases their susceptibility to blue mold. *Physiological and molecular plant pathology,* 30(3), 441-451.

Rossberg, M., Lendle, W., Pfleiderer, G., Tögel, A., Dreher, E. L., Langer, E., Rassaerts, H., Beck, U., Lipper, K. A., Torkelson, T.R., Löser, E., Beutel, K. K., y Mann, T. (2006). "Chlorinated Hydrocarbons" in Ullmann's Encyclopedia of Industrial Chemistry, *Wiley-VCH,* Weinheim. doi:10.1002/14356007.a06_233.pub2.

Sallaud, C., Giacalone, C., Topfer, R., Goepfert, S., Bakaher, N., Rosti, S., y Tissier, A. (2012). Characterization of two genes for the biosynthesis of the labdane diterpene Z-abienol in tobacco *(Nicotiana tabacum)* glandular trichomes. *The Plant Journal,* 72(1), 1-17.

SCOEL recommendations from The Scientific Committee on Occupational Exposure Limits (SCOEL) for Methylene Chloride (Dichloromethane). SCOEL/SUM/130, June (2009). European Commission Employment, Social Affairs & Inclusion Health and Safety at work. p. 38. Consultado 22-07-2014.

Seo, S., Gomi, K., Kaku, H., Abe, H., Seto, H., Nakatsu, S., Neya, M., Kobayashi, M., Nakaho, K., Ichinose, Y., Mitsuhara, I., y Ohashi, Y. (2012). Identification of Natural Diterpenes that Inhibit Bacterial Wilt Disease in Tobacco, Tomato and Arabidopsis. *Plant and Cell Physiology,* 53(8), 1432-1444.

Severson, R. F., Arrendale, R. F., Chortyk, O. T., Johnson, A. W., Jackson, D. M., Gwynn, G. R., Chaplain, J. F., y Stephenson M. G. (1984). Quantitation of the major cuticular components from green leaf of different tobacco types. *Journal of Agricultural and Food Chemistry,* 32(3), 566-570.

Shepherd, R. W., Bass, W. T., Houtz, R. L., y Wagner, G.J. (2005). Phylloplanins of Tobacco Are Defensive Proteins Deployed on Aerial Surfaces by Short Glandular Trichomes. *The Plant Cell,* 17(6), 1851–1861.

Shobha, S. V., y Kale, R. D. (2008). *In vitro* Studies on Control of Soil-Borne Plant Pathogens by Earthworm *Eudrilis Eugeniae* Exudates. *Green Pages*. http://www.eco-web.com/edi/080106.html. Consultado 26-01-2014.

Tissier, A. 2012. Glandular trichomes: what comes after expressed sequence tags?. *The Plant J.,* 70(11), 51-68.

USDHHS. US Department of Health and Human Services. Toxicological Profile for Methylene Chloride. Agency for Toxic Substances and Disease Registry. September (2000). Division of Toxicology/Toxicology Information Branch. p. 269. Consultado 16-07-2014.

Valgas, C., Machado de Souza, S., Smânia, E. F. A., y Smânia, A. Jr. (2007). Screening Methods to determine Antibacterial Activity of Natural Products. *Brazilian Journal of Microbiology,* 38, 369-380.

Wagner, G. J. (1991). Secreting glandular trichomes: More than just hairs. *Plant Physiology,* 96(3), 675-679.

Wagner, G. J. (1999). Leaf Surface Chemistry In Tobacco: Production, Chemistry, and Technology; D. L. Davis and M. T. Nielsen, eds.; *Blackwell Science*: Cambridge, p. 292- 303.

Wagner, G. J., Wang E., y Shepherd R. W. (2004). New approaches for studying and exploiting an old protuberance, the plant trichome. *Annals of Botany,* 93(1), 3-11.

Wang, E., Wang, R., Deparasis, J., Loughrin, J.H., Gan, S., y Wagner, G. J. (2001). Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product- based aphid resistance. *Nature biotechnology,* 19(4), 371-374.

Wang, E., Hall, J. T., y Wagner, G. J. (2004). Transgenic *Nicotiana tabacum* L. with enhanced trichome exudate cembratrieneols has reduced aphid infestation in the field. *Molecular Breeding,* 13, 49-57.

Whitfield, M. B., (2004). Integrated Bioprocessing of Native and Engineered Components from Tobacco (*Nicotiana tabacum* L.).Thesis for the Degree of Master of Science in the Crops Science, Department at North Carolina State University.

Wollenberg, E., Dörsam, M., Dörr, M., Roitman, J. N., y Valant-Vetschera, K. M. (2005). Chemodiversity of Surface Flavonoids in *Solanaceae*. *Zeitschrift fur Naturforschung C-Journal of Biosciences,* 60(9-10), 661-670.