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Full Length Research Paper

Effectiveness of leaf essential oils of *Cymbopogon citratus* and *Ocimum urticifolium* in controlling *Phytophthora infestans* Mont. damaging Irish potato in Ruhengeri (Rwanda)

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An assay of antifungal activity of essential oils of *Ocimum urticifolium* and *Cymbopogon citratus* was conducted to assess their potentials in controlling potato mildew in Irish potato. The disc diffusion method was used to evaluate the zone of fungal growth inhibition while the oil-supplemented medium was used to evaluate the antifungal activity of these essential oils at various concentrations. These essential oils were used to cure the infected plants as *ex situ* investigation. The results revealed that the essential oils exhibit fungicidal activity against *P. infestans* at higher concentrations. *In vitro* antifungal activity was recorded with the minimum inhibitory concentrations ranging from 200 to 10 µl/l and from 600 to 400 µl/l for the essential oils of *O. urticifolium* and *C. citratus*, respectively. The essential oil of *O. urticifolium* was more efficient than the essential oil of *C. citratus and the ex situ* investigation succeeded in curing infected plants. The eugenol and citral were described to play the major role in imparting the antifungal activity to this oil. The fungistatic effect against *P. infestans* was observed. These results indicate the possibility of exploiting *O. urticifolium* and *C. citratus* in fighting *P. infestans* which causes heavy losses of Irish potato.

Key words: *Cymbopogon*, Fungicidal effect, fungistatic effect, Irish potato, leaf essential oils, minimum inhibitory concentrations, *Ocimum*, *Phytophthora infestans*.

INTRODUCTION

The majority of Rwandans (about 89% of the population) live on agriculture which contributes to 42% of the national gross domestic product (GDP) (Kerlan, 1992). In Rwanda, the Irish potato is the basic food with high production spanning from 60 to 65% (Gashabuka, 2005) and occupies 44% of lava bed (Fane, 2004). In Rwanda and elsewhere, the production of this crop encounters many limiting factors particularly the diseases and pests that occasioned its poor yield in quality and in quantity (Daayf et al., 2003; Rutayisire, 1984). These biotic factors affect its quality and quantity and the mildew marked the most this history and is the only one significant constraint of the potato since its introduction in Rwanda (Fontem et al., 2004; Rolot, 2001). It is caused by the fungus *Phytophthora infestans (Mont.)* which attacks any parts of the plant and at any stages of growth and causes significant pre-harvest-losses (Nshimiyimana, 2007; Pattnaik et al., 1997; Yashpal, 1993).

During last several years, the chemicals have been used to control various plants diseases. However, the resistance of the phytopathogens to these chemicals and the accumulation of the undesirable residues in the food chain and other harmful effects on natural ecosystems raised many discussions (Carol and Beagle, 2000;

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Figure 1. The sliced tuber showing symptoms of potato mildew to access the inner parts.

Pattnaik et al., 1997; Fontem et al., 2004). This last is known as the phenomenon of bio-accumulation (Hilan and Sfeir, 1998). Even with low dose, the presence of the toxic substances in the environment can represent a danger to the living organisms through the food chains. These last years, this challenge disputed the use of chemicals in plant diseases control and led the scientists to direct their research towards other alternatives. From then on, the use of the bio-pesticides has been promoted for integrated management since many chemical fungicides do not by themselves afford satisfactory levels of control. However, the use of microbial control in agriculture presents a great danger to non-targeted insects (Bernardo et al., 2008). As for the plant-based pesticides, the studies succeeded to combat various kinds of diseases and pests. They are abundant in nature and their use respects other species of organisms in the ecosystem. Among them, the essential oils are being tested in Rwanda by Institute of Scientific and Technological Research (IRST). These essential oils are actually the mixtures of more or less many components that, alone or in synergy, impart to these oils the biocidal properties (Hilan and Sfeir, 1998; Fontem et al., 2004; Kakana, 2005).

Thus, having the same agricultural and environmental problems in Rwanda, we carried out a research project leading to the use of essential oils of *C. citratus* and *O. urticifolium* as bio-products to provide the farmers with the alternative way to reduce the fall in potato production. This works consists in the *in vitro* evaluation of the antifungal effect of essential oils obtained by hydrodistillation of fresh leaves of *C. citratus* and *O. urticifolium* on *P. infestans* Mont.

MATERIALS AND METHODS

Biological materials

The harvesting of plant materials were done after the dew was removed by the morning sun. The leaves of *C. citratus* and *O. urticifolium* were collected from the garden of medicinal and essential oils plants of the Institute for Scientific and Technological Research (IRST) at Butare in Southern Province. The isolate of *P. infestans* were isolated from the tubers of Irish potato affected showing the symptoms of mildiew in the field of Rwanda Agricultural Board (RAB) in Kinigi, Northern Province (Figure 1).

Extraction of essential oils

For each species, the extraction of essential oils was carried out in natural products laboratory of IRST and was performed in Clevenger apparatus for 5 h on 100 g of leaves dried in cool place. The leaves were washed with fresh water and introduced in clean flask. The water was added to cover the mixture and the grains of pumice were introduced in to regulate the boiling and homogenize the temperature inside the flask. The flask was connected to the distillation system and the mixture was brought to boil. The organic phase was recovered by decantation method from hydro-distillate. This organic phase was the solution containing the essential oils we looked for. It was stored in hermetically sealed dark glass flask with rubber lids to protect it from light and kept in refrigerator at 4°C until use (Jazet et al., 2008; Koba et al., 2009).

Isolation of *P. infestans*

The fungus was isolated from infected tubers of two varieties of Irish potato (Gikungu and Sangema) and grown on agar plate. These varieties were chosen because they are the most endangered ones and the most preferred by the farmers. The collected tubers were packed and brought to the microbiology laboratory of the High Institute of Agriculture and Animal Husbandry (ISAE) at Busogo/Rwanda. The tubers were cleaned with distilled and sterile water and the inner parts were cut into small slices of 1 cm of diameter. These slices were simultaneously put on sterile Potato Dextrose Agar or PDA medium (Daayf et al., 2003) and Potato Count Agar or PCA medium contained in Petri dishes and the cultures were incubated at 30°C for 5 days. The two culture media were used in order to identify which one favors the fungi growth the most. Different colonies appeared and were biochemically and morphologically characterized. According to the characteristics of

appeared colonies, different colonies of *P. infestans* were spotted and isolated. In choosing the tissues, the newly infected and inner tissues were preferred in order to avoid the appearance of many types of microorganisms that should complicate the identification. After the isolation is finished, the *P. infestans* culture was maintained with periodic sub-culturing under the same growth conditions. The isolation was performed in duplicates.

Essay of *in vitro* antifungal activity

The isolated fungus was maintained in the culture collection of Microbiology Laboratory of the Institute of Agriculture and Animal Husbandry (ISAE-Busogo). Before the experiments started, the fungal suspension for *P. infestans* was prepared with an absolute asepsis using the spores of fungal hyphae. Using the spectrophotometer, the cells concentrations for this fungus were adjusted to 3.10^8 cells/ml. Thereafter, a quantity of each cellular suspension was transferred to the sterile flasks. Antifungal activity of the essential oils was evaluated by two methods: disc diffusion method and agar incorporation method. For the agar diffusion method, the test was performed in sterile Petri dishes containing PDA medium at the rate of 20 ml per dish.

The samples of essential oils were dissolved in ethyl acetate and the same quantity of aliquots was applied to sterile paper discs, at the rate of 10 µl of solution per disc. The discs were placed on the surface of the culture medium inoculated with a fresh fungal suspension (Terezinha de Jesus et al., 2006). The Petri dishes were then incubated at 30°C to allow the growth after the measurement of inhibition zone (diameter) was performed. As for oil-supplemented agar, we referred ourselves to Jazet et al. (2008). Essential oils were mixed with dimethylsulfoxide (DMSO) in proportion of 1:9 to facilitate its solubilization in the sterilized PDA medium. The mixture of essential oil/DMSO was incorporated in the PDA medium in desired concentrations: initially 5000, 4000, 3000, 2000, and 1000 µl/l. The concentrations were lower than those inhibited by the fungal growth viz., 800, 700, 600, 500, 400, 300, 200 and 100 µl were used for the search of the minimal inhibitory concentration (MIC). The supplemented medium was poured into Petri dishes and allowed to rest for solidification. These two tests were carried out in triplicate and average of measures was used as results.

Test of antifungal activity on plants deliberately infected

The *in vitro* plantlets of one variety of Irish potato locally named Gikungu were obtained from *in vitro* tissue culture laboratory of Rwanda Agricultural Board (RAB) and the same fungal strain used *in vitro* were used for this test. The healthy 30 plantlets planted in culture medium contained in transparent and plastic bags were deliberately inoculated with *P. infestans* and incubated at room temperature until the appearance of fungal colonies. After the symptoms clearly appeared, the essential oil was applied on infected parts with sprayer. The disease evolution was monitored by the macroscopic observations with positive control in support.

Figure 2. Three-day old culture of P. infestans on PDA medium. The medum favoured the development of hyphae which finally give blackish spores.

RESULTS

After 5 days of incubation of infected vegetative parts on culture media, the whole surface of PCA medium was invaded by whitish colonies of mycelia while PDA medium favoured the development of hyphae culminated by black spores. During the essential oil extraction by hydrodistillation method and from 4 kg of dried leaves of each plant, the yield rates were 0.43 and 0.22% for *O. urticifolium* and *C. citratus* respectively (Figure 2).

In using the discs impregnated by oils and the medium supplemented with essential oils, both the essential oils showed the antifungal activity against *P. infestans*; however, the essential oil of *O. urticifolium* was more efficient. By disc diffusion method (DDM), the inhibition of growth was excellent with the oil of *O. urticifolium* (inhibition zone of 33 mm of diameter) on PDA medium and the inhibition was less with *C. citratus* oil (inhibition zone of 11 mm of diameter) on the same culture medium. However, out of the zone of inhibition caused by the essential oil of *O. urticifolium*, the colonies of the fungus appear but they do not develop properly.

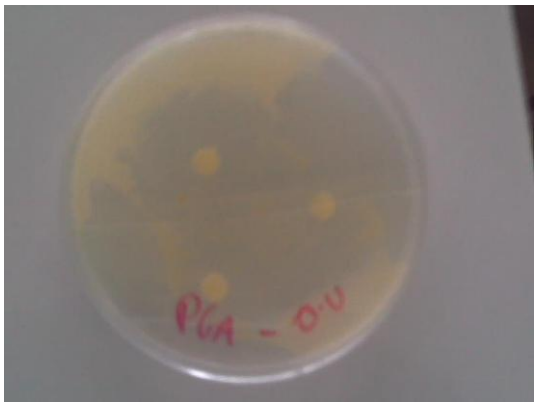

Figure 3. Inhibition zones caused by the essential oil of O. urticifolium.

Figure 5. The oil-treated in vitro plants (punnet A, left) and non-treated vitroplants (punnet B, right).

Figure 4. Inhibition zones caused by the essential oil of C. citrates.

By agar incorporation method (AIM), on the medium in which the essential oils were incorporated, the results were also meaningful. On both the cultures, the essential oils in all concentrations inhibited totally with the growth of *P. infestans* and no fungus colonies appeared in these cultures. In search of minimal inhibitory concentration (MIC) of each type of essential oils concerned by this study, we observed that it was within the range of 100 to 200 µl/l for the essential oil of *O. urticifolium* and 600 to 500 µl/l for the essential oils of *C. citrates* (Figures 3 and 4).

The plantlets of potato from *in vitro* culture inoculated by *P. infestans* showed the first symptoms on the third day. These symptoms appeared as white fungal colonies on stem and leaves, the black color on stem's nodes and leaf axils due to the necrosis of infected tissues and the appearance of light spots on underside of leaves. The application of essential oils to the infected parts of the plants resulted in colony disappearance on 12th day of treatment (Figure 5).

DISCUSSION

The results of this study have got meaningful explanations. The fact that our cultures gave only one microbial type means that the use of inner plant tissues is the reliable method for successful fungus isolation. As we wanted adequate form of fungus for easy cell suspension preparation, the fungal spores served as appropriate materials and for this reason, the PDA medium was chosen to be an appropriate medium for our study. The appearance or absence of fungal colonies by the two methods, DDM and AIM (Materials and methods), found the explanation from the chemical composition of the essential oils in use that we learned about other authors. In fact, the majority of essential oils have both the light and heavy molecules. Under normal conditions, the light molecules of essential oil volatilize easily whereas the heavy molecules volatile hastily. The method of discs allowed the fraction made of light molecules to circulate in free space inside the Petri dishes while the fraction made of heavy molecules stayed lying on the surface of the culture medium (Jazet et al., 2008; Koba et al., 2009). The heavy molecules of essential oils of *C. citratus*, alone or in synergy with the light molecules exhibited an antifungal effect against *P. infestans*. For the essential oil of *O. urticifolium*, the fraction of its essential oil made of light molecules exhibits a fungistatic activity while the part made of heavy molecules has fungicidal activity against the pathogen. However, the characterization of antifungal activity of these two fractions of the essential oils of *O. urticifolium* and *C. citratus* would require specific studies further. The absence of total fungus growth testifies the antifungal activity of both the essential oils of these two plant species is qualified to be fungicidal. The results

obtained by these two methods led us to establish interesting hypotheses on these essential oils. In fact, the light components, alone or in synergy with heavy components, would impart a lethal or fungicidal effect to the essential oil of *C. citratus* and *O. urticifolium*. For the final knowledge about these hypotheses, further and deep studies on different fractions are necessary for final conclusion.

For the essential oils obtained by hydrodistillation, some of their respective components have been identified earlier by Ntezurubanza (2000). In this process, the mixture of fresh water and plants vegetative parts is put in a flask and brought to boil. The steam passes upward through plant vegetative causing the evaporation of volatile and fragrant chemical compounds. In this case, the plant cells bust and release the chemical species (soluble or non soluble in water) which are driven by water vapor through condensation coil and finally recovered in other container after the cooling process made with water passing across the refrigerator. The obtained hydrodistillate is made of the aqueous phase and organic phase (essential oil) which are often clearly separated due to their different density. From what we learn from previous extraction works (Vieira et al., 2001; Terezinha de Jesus et al., 2006), the results we obtained during the extraction of these essential oils were reliable and this assure that our oil extraction were successful.

In this work, it is obvious that the antifungal potential of essential oils of *O. urticifolium* and *C. citratus* against *P. infestans* is a predictable consequence of some of its high concentrated components. A survey of the available literature showed that the antimicrobial effect of some essential oils components has been investigated. Hilan and Sfeir (1998) and Mahmoud (1999) found that geraniol and eugenol were effective in suppressing most of fungi strains growth while Viollon and Chaumont (1994) and Onawunmi et al. (1989) reported that antifungal activities of citral (geranial+neral), geraniol and citronellol showed the highest antifungal activities. The study of Ntezurubanza (2000) showed that the essential oil of *C. citratus* contained a high amount of citral (82%) while the eugenol was the major constituents of the essential oil of *Ocimum* genus including of course *O. urticifolium* (93%) (Vieira et al., 2001; Komoun, 2000). The marked action of alcohols in these oils may be attributed to the polarity of OH- group making these compounds relatively soluble in water and the terpenoids moiety confers lipophilic properties on these molecules with the ability to penetrate the plasma membrane (De Billebeck et al., 2001; Knobloch et al., 1989).

From our results and information about other essential oils' MICs from various investigations, it can be concluded that the essential oils of *O. urticifolium* and *C. citratus* exhibit a strong antifungal activity against *P. infestans* responsible for causing heavy losses on Irish potato. With this experiment, it is understandable that these essential oils could be used as easily accessible source of natural antifungal agents against this fungus. However, for the practical use of these oils as novel fungal control agent, further research on the formulation is needed to improve the fungicidal potency and stability.

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