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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (6): 928-932, 2000

Efficacy of Antimicrobial Plant Crude Extracts on the Growth of *Colletotrichum gloeosporioides* f. sp. *manihotis*

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Abstract: This study was conducted to investigate a cheap and readily available alternative control measure for cassava anthracnose disease causal agent (*Colletotrichum gloeosporioides* f. sp. *manihotis*), through the use of antimicrobial crude plant extracts such as neem (*Azadirachta indica*), bitter leaf *(Vernonia amygdalina), Ocimum gratissimum* and *Xylopia aethiopica,* on the fungal growth parameters (mycelial growth, sporulation and germtube development). The extracts at concentration levels of 25, 50, 75 and 100% full strength concentration showed an inhibitory effect on mycelial growth, germ tube development and sporulation of fungal isolates 05FCN, 10FCN, 12FCN and 26FCN. Neem seed and leaf extracts at 100% showed a total reduction in sporulation in most of the fungal isolates. The inhibitory properties of the plant crude extracts indicated a promising control option for consideration in cassava treatments of planting stocks, particularly in areas where farming is at a basic subsistent level, and less available money to meet the high cost of pesticides.

Key words: Antimicrobial, *Colletotrichum*, pant extracts

Introduction

The fungus *Colletotrichum gloeosporioides* f. sp. *manihotis* is the causal agent of cassava anthracnose disease (CAD). The fungus is best characterized by their relatively short straight cylindrical to broadly ellipsoidal conidia, with obtuse ends and truncated attachment point (Baxter *et al*., 1983). CAD is one of the most important cassava diseases frequent in the cassava growing regions of Africa (Hahn *et al*., 1989), South America (Lozano *et al*., 1981) and Asia (Nair *et al*., 1979). CAD in most cases often occurs in association with cassava bacterial blight, and symptoms of the two diseases are sometimes difficult to distinguish in the field (Theberge, 1985).

The importance of the joint action of stem puncture by an insect vector (*Pseudotheraptus devastans* Dist) and infection by the fungus *C. gloeosporioides* f.sp *manihotis* in the spread and development of CAD has been reported (Makambila and Bakala-Koumouno, 1982; Boher *et al*., 1983). The CAD fungus attacks tissues weakened by the insect puncture and lytic action of its saliva. This induces dark-brown lesions which can either remain limited or become deep-brown cankers with lacerated epidermis (Theberge, 1985). CAD in some cases spread and causes tip-dieback, leaf wilting, defoliation, petiole epinasty and poor sprouting (Lozano and Booth, 1974).

Accurate assessment of yield loss due to CAD has not been evaluated. However, in 1975, up to 90% of the local cassava cultivars of Zaire were rated severely infected (PRONAM, 1977). CAD severity could lead to a significant loss in planting materials and a decrease of 20-45% germination. In some cases severely affected stems result in a delay in sprouting by 7-8 days, as compared to disease-free planting materials and total crop failure (Van der Bruggen *et al.*, 1990; Ikotun and Hahn, 1991).

Little attention has been made in the development of control measures for CAD due to the fact that the causative agent *C. gloeosporioides* f.sp *manihotis* has been considered a weak pathogen (Van der Bruggen and Maraite, 1987). However, increased incidence and severity of CAD in both farmers and research fields has led to investigation of possible control measures. Cultural control measures aimed at reducing the spread of CAD pathogen, such as crop rotation deep burial of infected plant debris and manipulation of planting time has been reported (Lozano *et al*., 1981; Fokunang, 1995). The use of resistant varieties of planting materials has also been recommended as the most efficient means of controlling CAD (Muimba *et al*., 1983; IITA, 1990). Progress in the establishment of resistant variety is slow due to the duration it takes to breed for durable or polygenic genotypes for CAD resistance. Thus, breeding for resistance is an efficient but long term control strategy, which can not serve the short-term needs of cassava growers.

Chemical control measure for CAD has not yet been well established. However, Lozano *et al*. (1981) and Theberge (1985) suggested the use of copper fungicides in cases where resistant stocks were not available. Further investigation in the area of chemical control has been slow due to the rising cost of production and sales of the limited fungicides available in the market, which the poor subsistent farmers are unable to afford. Most synthetic fungicides are non-biodegradable, phytotoxic and pose problems to non-target organisms and their environment (Naidu and John, 1981; Birch *et al*., 1993).

Further attempts in the investigation into farmers problem of disease control has been focused in the use of anti-microbial plant products for pre-treatment of planting material. These natural plant products are not only readily available to the farmers, they are biodegradable, non-phytotoxic, environmentally friendly and very promising within the frame work of integrated pest management systems (IPMS) (Awuah, 1989; Mukerji *et al*., 1992). Fungitoxic, fungistatic and fungicidal activity of plant extracts on plant pathogens has been widely reported (Naidu and John, 1981; Tripathi *et al*., 1985; Awuah, 1989; Shetty *et al*., 1989). The effectiveness of the plant extracts depends on the nature and amount of active principle it contained. Plant extracts containing stronger and higher levels of antifungal principles are effective even at lower dilutions (Nene and Thapliyal, 1965).

The aim and objective of this study was to investigate the efficacy of antimicrobial plant product on the growth of *Colletotrichum gloeosporioides* f. sp. *manihotis* isolates. The use of antimicrobial products from neem *(Azadirachta indica*), bitterleaf (*Vernonia amygdalina*), *Ocimum gratissimum* and *Xylopia aethiopica* also aimed at establishing a cheap and affordable alternative control measures for cassava anthracnose disease, a major economic disease in of cassava in Africa.

Materials and Methods

Preparation of plant crude extracts: The extracts of neem, bitter leaf, *Xylopia* and *Ocimum* were prepared by crushing the sun-dried leaves and seeds after washing thoroughly with sterile distilled water. The materials were dried at room temperature ($25 \pm 2^{\circ}C$ for 72 hours before extraction. The leaf and seed extracts were prepared by crushing 100 g of dried material with 300 ml of sterile distilled water. The extracts were filtered through a 3-layered muslin cloth. The oil from neem seeds was obtained with the aid

of a soxhlet extractor. The extracts were sterilized heat-free by passing them through a milipore of 0.22 µm pore size using a Swinner filter adaptor. The supernatant was stored at 10°C in a refrigerator for future use.

Collection, isolation and identification of the pathogen: Cassava stems showing symptoms of anthracnose infection (dark brown lesions, deep cankers) were collected from infected fields. Small pieces of infected materials were cut from advancing edges of the canker, surface-sterilized for approximately 3-5 minutes in 10% sodium hypochlorite solution, rinsed in five successive changes of sterile distilled water. The stem pieces were dried on sterilized filter papers and placed on Potato Dextrose Agar containing 100 mg/dm^3 sodium novobiocin (PDA + N). The material was incubated at 25°C under intermittent mixed irradiation for 12 h/day from near ultra-violet and daylight type of fluorescent tubes (Philips T1 40W/8R5, F40 BLB and TL 40W/33R5 respectively), suspended 50cm above the plates. Isolation of the fungal isolates was made on PDA + N and 9cm Pyrex Petri dishes were used throughout. Identification of the fungus was done under the microscope and confirmation made using the procedure of Barnett and Hunter (1972).

***In vitro* investigation of antimicrobial properties of crude plant extracts in fungal growth**.: The crude extracts were used at different concentrations of (100, 75, 50 and 25%), prepared by serial dilution of the stock solution extracts, in the ratio of (1:2:3:4 ml of distilled water) respectively. Their antimicrobial properties were investigated on five virulent isolates of *C. gloeosporioides* f.sp*. manihotis* (05FCN, 10FCN, 12FCN and 26FCN). The control treatment was made of an extract-free PDA media.

Concentration treatment contained four replications arranged in a randomized complete block design. Plates with PDA + N were inoculated with 5 mm-diameter mycelial disc of isolates and incubated for 48 h under alternate 12 h-light/12 h dark condition and a temperature of 25 ± 2°C for growth initiation. Each isolate consisted of five replicate plates arranged in a randomized complete block design.

For mycelial growth parameter measurement, flame-sterilized 5mm cork-borer mycelial discs were randomly cut out from an 8-day old pure culture of the fungal isolates. Each of the mycelial discs was aseptically transferred with the aid of a flamed-sterilized and cooled mounted needle to the centre of a molten PDA media contained in Petri dishes, the bottom of which was marked with two perpendicular lines passing through the centre. The plates were incubated at 25 ± 2°C and the mycelial growth was measured along the perpendicular lines and the mean was calculated for each treatment. Records of mycelial growth of the fungal isolates were taken at 3, 5, 7 and 9 days after growth initiation.

For sporulation density measurement, spore suspensions were prepared from 7-day old cultures of the fungal isolates by flooding with sterile distilled water and dislodging the spores with a small brush. The suspension was centrifuged at 2000 rpm for 3 mins and the supernatant was filtered through 2 layers of sterile muslin cheesecloth. A drop of the resulting spore suspension was placed on a haemocytometer chamber covered with a cover slip and the number of spores/ml of sterile distilled water was estimated as the average of spores counted in 10 large squares (Standard haemocytometer fields). Sporulation density was calculated using the formula adopted from Tuite (1969): S = NV/v; where S = number of spores/ml; N = mean number of spores in 10 large squares counted; V = 1 ml = 1,000 mm^3 and n = volume of spore suspension under glass cover.

Spore germination was recorded using standard procedures described by Kiraly *et al*. (1974). One hundred spores were observed for spore germination at 8 h and 16 h intervals and germination was based on microscopically visible germ-tube formation.

Statistical analysis: The data for mycelial growth, sporulation and spore germination were subjected to analysis of variance (ANOVA) (SAS Institute, 1989). Fischer-protected LSD test of mean separation was performed only when the ANOVA showed significance.

Results

Effect of crude plant extracts on mycelial growth, sporulation and spore germination of fungal isolates: Crude plant extracts at different concentration levels showed variation in the reduction of growth of *C. gloeosporioides* f. sp*. manihotis* isolates 05FCN, 10FCN, 12FCN and 26FCN (Fig. 1). There was significant reduction in fungal growth parameters in all crude extract treatments above 50% concentration. Neem leaf and seed at 100 % full strength concentration showed a total reduction in mycelial growth in all fungal isolates (Fig. 1). There was significant reduction in spore germination of fungal isolates in all crude extract treatments at 75 and 100% concentration (Fig. 2). Neem seed Ocimum and neem leaf showed a total reduction in spore germination at full strength concentration.

There was significant variation in sporulation density of isolates at the different crude extract concentration levels (Fig. 3). At full strength concentration of crude extract treatments, there was total reduction in sporulation in most of the fungal isolates, treated with bitter leaf, neem seed, *Ocimum*, neem leaf and *Xylopia* (Fig. 3). Crude plant extracts treatments showed variation at the different concentration levels. Neem seed at 50 and 100% showed an overall maximum inhibition of mycelial growth spore germination and sporulation (Table 1). Least mycelial growth was recorded with neem leaf extract treatment at 50 and 100% concentration, with mean growth of 20.44 mm diameter and no growth respectively. *Xylopia* extract recorded the highest mycelial growth, with least inhibition of mean value of 41.81mm, at 50% and 17.13 mm at 100%. Bitterleaf extract also recorded low sporulation of 6.9×10^5 spores/ml of distilled water and spore germination of 2.19%, at full strength concentration. Spore germination was maximum in *Xylopia* extract at both half and full strength with mean values of 27.94 and 4.38% respectively.

Crude extracts at 50 and 100% concentration showed maximum inhibition of mycelial growth of fungal isolate 05FCN (Table 2). Overall maximum growths of mycelia for both concentrations were 27.15 mm and 4.50 mm respectively. Sporulation was least with isolate 12FCN at 100% concentration with mean value of 5.5×10^5 spores/ml of sterile distilled water. Fungal isolate 05FCN showed an overall total inhibition of spore germination at 100 % crude extract concentration.

Discussion

Investigation of the antimicrobial properties of crude plant extracts on the growth parameters of *C. gloeosporioides* f. sp *manihotis* isolates showed that crude plant extracts possess some inhibitory components which can cause significant reduction in mycelial growth, sporulation, and spore germination of the fungus. Neem seed extract and neem leaf at 100% concentration showed total reduction in sporulation of most of the isolates. In all the extracts at different concentrations, neem leaf, neem seed and *Ocimum gratissimum* extracts showed promising fungitoxic properties at above 50% concentration.

Fungitoxic and fungicidal properties of plant extracts on plant pathogens has been widely investigated. Akpa *et al*. (1991) reported that fresh leaves and fruits of neem plants showed a significant inhibitory property on mycelial growth of sorghum anthracnose disease, *Colletotrichum graminicola*. Awuah (1989) also found that extracts from *Ocimum gratissimum* led to 24.6% reduction in radial growth of *Rhizopus spp* and a 60% reduction of *Ustilaginoidea virens*. He also showed that *Xylopia aethiopica* had strong fungitoxic properties by inhibiting the growth of *Rhizopus spp* and *Ustilago maydis*, but was not effective against *U. virens*.

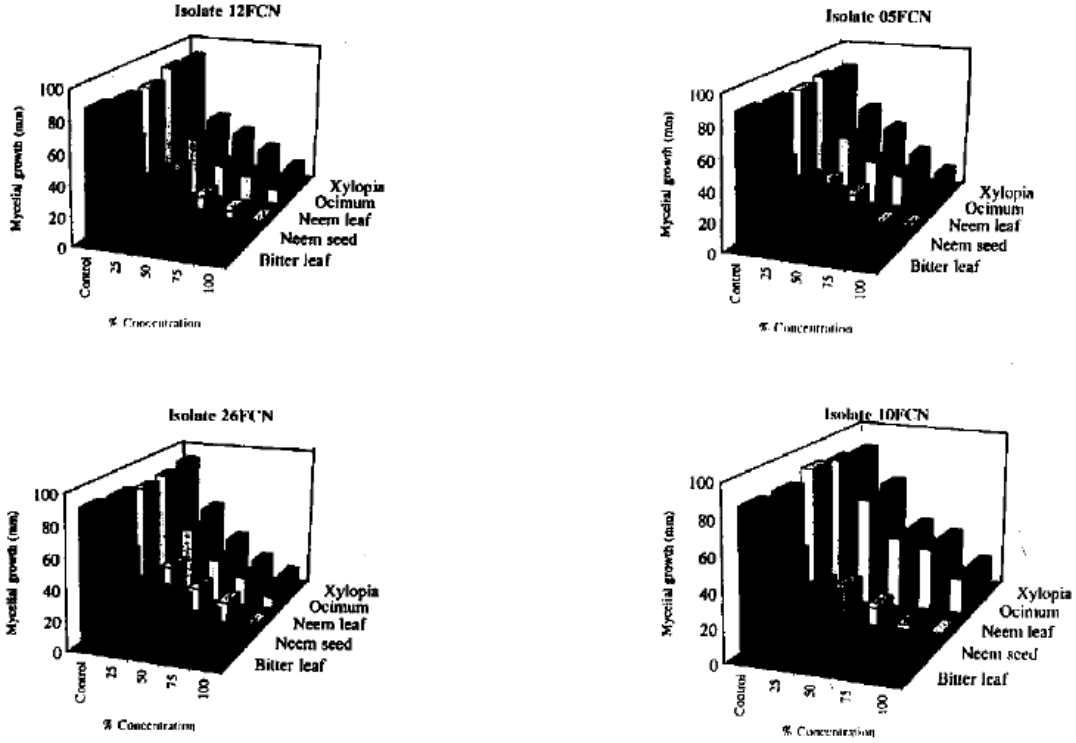

Fig. 1: Effect of crude plant extracts on mycelial growth of *C. gloeosporioides* f. sp. *manihotis* isolates

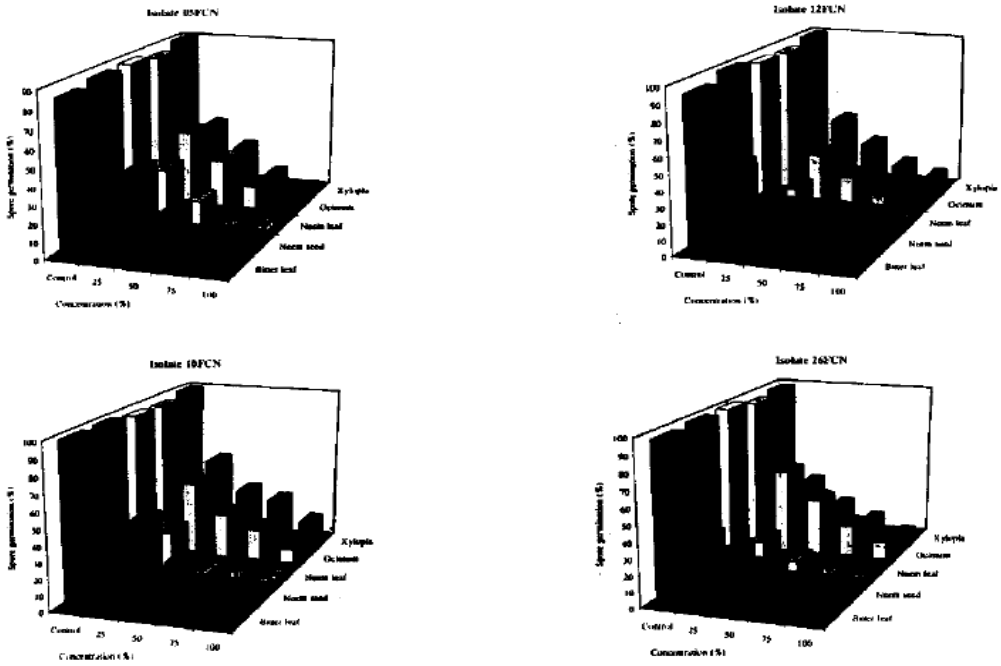

Fig. 2: Effect of crude plant extracts on spore germination of *C. gloeosporioides* f.sp. *manihotis*

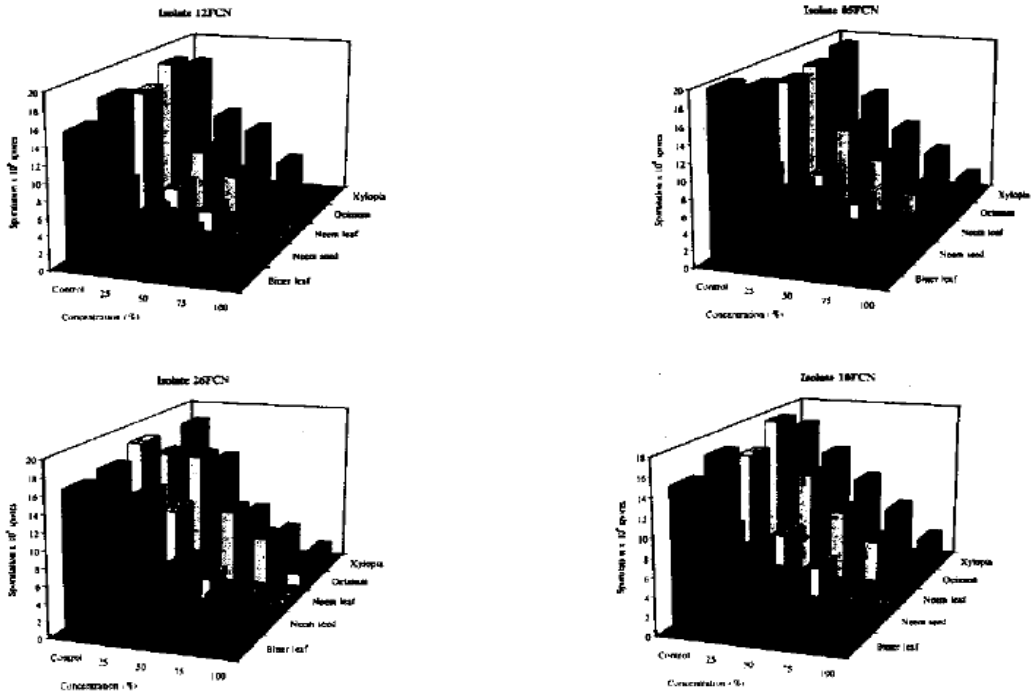

Fig. 3: Effect of crude plant extracts on sporulation of *C. gloeosporioides* f.sp. *manihotis*

Table 1: Overall effects of crude plant extracts at 50% and 100% concentration on growth parameters of fungal isolates.

Crude extracts	Mycelial growth/mm		Sporulation $\times 10^6$ spores/ml		% Spore germination	
	50%	100%	50%	100%	50%	100%
Xylopia	41.81a	17.13a	5.54a	1.69a	27.94a	4.38a
Vernonia	41.69a	11.75b	5.25a	0.69c	21.88c	2.19b
Ocimum	25.25b	6.87c	4.89a	1.28b	26.02b	4.94a
NSE	10.50d	1.25d	1.39b	0.00d	4.81e	0.00c
NLE	20.44c	0.00e	3.13ab	0.00d	13.94d	

0.00cNSE = Neem seed extract; NLE = Neem leaf extract

Means in the same column followed by the same letter(s) are not significantly different at $p \leq 0.05$ by Duncan Multiple Range Test

Table 2: Overall reaction of fungal isolates to two concentration levels of crude plant extracts.

Isolate	Mycelial growth/mm		Sporulation $\times 10^6$ spores/ml		% Spore germination	
	50%	100%	50%	100%	50%	100%
05FCN	27.15c	4.50d	4.50a	0.26	18.45c	0.00d
10FCN	28.85b	11.10a	3.39a	1.15a	23.10a	5.05a
12FCN	22.35d	5.75c	3.87a	0.55b	12.95d	1.52c
26FCN	33.40a	8.25b	4.49a	0.97a	21.20b	3.00b

Means in the same column followed by the same letter is not significantly different at $p \leq 0.05$ according by Duncan Multiple Range Test

Aqueous extracts from leaves, barks and seeds of *Azadirachta indica*, *Ocimum basilicum*, and *Vernonia negundo* (Bhowmick and Verdhan, 1981), were found to have significant inhibitory effect on *Trichoconiella padwickii*, a seed-borne fungus of paddy rice. The effectiveness of plant extracts depends on the nature and amount of active ingredient it contained. The fungitoxic properties in neem extracts has been attributed to azadirachtin, azadirachtol and nimbin (Warthen *et al*., 1984), xylopic acid for *Xylopia aethiopica* (Oji *et al*., 1992), vernodalin and vernomydalin for bitter leaf (Kupchan *et al*., 1969) and the thymol derivatives for *Ocimum gratissimum* (Tripathi *et al*., 1985). Some studies have attributed neem control of plant fungi to the sulphur compounds present in the neem seed (Pant *et al*., 1986). Investigation of the effect of leaf extract media of 23 plants including *Azadirachta indica* and *Ocimum sanctum* by Tewari and Dath (1984) on the radial growth and sporulation/sclerotia production of *Pyricularia oryzae, Drechslera oryzae* and *Corticium sasaki* showed a broad-spectrum fungitoxicity in the oil extracts. This fungitoxicity could be compared to some synthetic fungicides like Blitox 50, Brassicol, Dithane Z-78, Aureofungin and Difolatan.

The report by Singh *et al*. (1980) confirmed the fungicidal properties of *A. indica* on some soil-borne pathogenic fungi such as *Fusarium oxysporum*, *Rhizoctonia solani, Sclerotium rolfsii* which caused wilt and rot in *Cicer arietinum*. There is the potential for these crude plant extracts to become local source of natural fungicides due to the fact that they are low cost raw materials with low mammalian toxicity, and are easily biodegradable in the soil. The most desirable attribute of the crude plant extracts is their environmentally sound pest and disease control agent within the framework of integrated pest management systems (IPMS).

Acknowledgements

We thank the Training Program, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria for the financial assistance of this work, and the Tuber and Root Improvement Program (IITA) for their technical assistance. The anonymous reviewers of the manuscript are also gladly acknowledged.

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