PHYTOCHEMICAL ANALYSIS AND ANTIFUNGAL ACTIVITY OF GYMNOSPERM AGAINST *FUSARIUM* WILT OF BANANA

AIN, Q. U.[1*] – ASAD, S.[2] – JAMAL, A.[2] – ARIF, M.[3] – MAHMOOD-UL-HASSAN, M.[4]

[1]*PARC Institute of Advanced Studies in Agriculture, National Agricultural Research Center, Islamabad, Pakistan*

[2]*Crop Diseases Research Institute, National Agricultural Research Centre, Islamabad, Pakistan*

[3]*Plant Pathology Department, The University of Agriculture, Peshawar, Pakistan*

[4]*Land Resources Research Institute, National Agricultural Research Centre, Islamabad, Pakistan*

[*]*Corresponding author*
e-mail: quratulainsatti@yahoo.com; phone: +92-344-810-7441

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Abstract. Fusarium vascular wilt caused by hyphomycete i.e. *Fusarium oxysporum* f. sp. *cubense* (Foc) exterminated the banana industry of Central America due to its dependence on monoculture. In this investigation, six gymnosperm plants i.e. *Cupressus sempervirens*, *Thuja orientalis*, *Cedrus deodara*, *Pinus wallachiana*, *Picea smithiana* and *Pinus roxburghii* were evaluated for their ability to control Foc; Tropical race 4 (TR4). Leaf and cone extracts (5-10%) of all six plants were screened using poisoned food technique. Extracts recording higher percent inhibition (≥60%) were further evaluated, an *in vitro* and a pot experiment. Highest percent inhibition in the *in vitro* assay and lowest disease severity index (DSI) in the pot experiment were recorded for ethanol leaf extract of *P. wallachiana* (Pw_{EL}), followed by *T. orientalis* ethanol leaf extract (To_{EL}). The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of both extracts was performed and the results indicated detection of seven compounds. Three of the compounds *viz* cis-9-Hexadecenal, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid were found in both extracts whereas Vitamin C, Hexatriacontyl trifluoroacetate, Oleic acid, delta-Cadinene were found in Pw_{EL} while Cedrol, Podocarp-7-en-3-one, 1-Phenanthrenecarboxylic acid and Pimaric acid were identified in To_{EL}. It is hypothesized that these phytocompounds might be the possible reason of Foc inhibition in current studies.

Keywords: *Fusarium oxysporum f. sp. cubense*, *poisoned food technique*, *disease severity index*, *Gas Chromatography-Mass Spectrometry*, *phytocompounds*

Introduction

Banana belongs to *Musa* genus and Asia is its center of origin (Ploetz et al., 2007). Trade quality banana are parthenocarpic and triploids that are vegetatively propagated (Simmonds, 1986). Even though hundreds of banana cultivars exist globally, majority of the production is based on few cultivars (Perrier et al., 2011). Panama epidemic devastated banana industry of Central America that was exclusively growing susceptible variety Gros Michel (Drenth and Guest, 2016). There was a major substitution of susceptible banana with resistant Cavendish cultivars (Ghag et al., 2015). Due to dissemination of diseased rhizomes and suckers intercontinental spread of the fusarium wilt occurred. Fusarium wilt is reported in most of the banana growing regions of the world e.g. Australia, Asia, Tropical Americas and Africa. The disease is induced by a hyphomycete named *Fusarium oxysporum* f. sp. *cubense* (Ploetz, 2000, 2006). External disease symptoms consist of leaf yellowing of older leaves, pseudostem splitting followed

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by leaf buckling and wilting (Yin et al., 2011). Internal symptoms consist of vascular discoloration blocking water conducting xylem vessels (Mackesy and Sullivan, 2015).

There are four recognized races of Foc separated on the basis of host susceptibility. Race 4 attacks Cavendish cultivars as well as those varieties that are attacked by race 1 and 2. Race 4 has two recognized strains i.e. TR4 (tropical race 4) and SR4 (subtropical race 4). The SR4 attacks banana that are growing suboptimally while TR4 attack Cavendish banana under any growing conditions (Ploetz, 2015; Drenth and Guest, 2016). Intercontinental spread of infected rhizome bits and suckers has led to the transmission of Foc TR4 from Taiwan to China, Indonesia, Philippines, Malaysia, Myanmar, Australia, Laos, India, Pakistan, Oman, Lebanon, Israel, Jordan and Mozambique. Dissemination of TR4 is much quicker (Drenth and Guest, 2016; Dita et al., 2018).

In Pakistan Fusarium wilt was reported in small farm, Thattha district in 2012-13 (Syed et al., 2015). Pakistan banana industry upto 95% of the area is based on Dwarf Cavendish (Basrai) cultivation. Presence of Foc TR4 is an alarming situation that if not controlled could lead to devastation as observed in past. Cultural practices i.e. flood-fallowing, suppressive soil, soil solarization and crop rotation are proposed (Stover, 1962) but have limited control (Gnanasekaran et al., 2015; Pegg et al., 2019). Development of resistant cultivars from diploids that are seed bearing is very expensive and time consuming (Ortiz and Swennen, 2014).

Different control options have been seeked in the recent decades for Foc management using chemicals, antagonistic microbes and botanicals against Foc. These management studies reported that control comes either by directly affecting the Foc morphology or by stimulating the host response. That is achieved from chemical compounds that might be the fungicide or the metabolites of plants and microbes. Many research investigations comprising on the efficacy of botanicals indicated that the secondary metabolites of higher plants possess such phytochemicals that had antimicrobial activity against phytopathogens (Doughari et al., 2009; Saravanakumar et al., 2015). Gymnosperms have never been tested against Foc although their antimicrobial activity is well known. This study addressed wilt problem using gymnosperm botanicals as control option for substituting conventional fungicide use. Six gymnosperm plants were selected including *Cupressus sempervirens*, *Cedrus deodara, Picea smithiana*, *Pinus wallachiana, Pinus roxburghii* and *Thuja orientalis*.

Materials and methods

Acquisition, revival and confirmation of fungal culture

Fusarium oxysporum f. sp. *cubense* (Foc; TR4) was graciously provided by the Tissue culture lab at National Agricultural Research Centre (NARC), Islamabad. Its molecular confirmation tests had already been performed by tissue culture department that isolated Foc from the diseased banana rhizomes collected from Thatta district of Sindh, Pakistan (Muhammad et al., 2017). The Foc culture was revived on Potato Dextrose Agar (PDA) and its morphological characters were analysed. Microscopic studies showed typical conidial morphology of Foc TR4. Microconidia are kidney shaped, hyaline, produced on false heads, mostly without septation. Macroconidia were sickle-shaped, hyaline, pointed at both ends, mostly 3-5 septate, borne on single phialides.

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Collection and drying of samples

Fresh leaves and cones of selected botanicals were collected from Murree (33°54′15″N 73°23′25″E with 2291 m altitude) and Swat (35°12′N 72°29′E with 980 m altitude) in the year 2014 and brought to fungal pathology lab at Crop Diseases Research Institute (CDRI), NARC, Islamabad, Pakistan. The samples were thoroughly washed and disinfected with 5% Clorox. Collected samples were kept under shade for one month. Dried samples (leaf and cone) were ground in an electric grinder and stored in labeled air tight containers till further use.

Extraction of samples

The n-hexane, ethanol and methanol were used as solvents for extraction. Two concentrations (5% and 10%) for each sample were prepared (Monteiro et al., 2013). Powdered samples were individually mixed with 100 mL solvent in flasks (Erlenmeyer) and shaken at 60 rpm (revolution per minute) for 48 hours. Extract in each flask was filtered and filtrate's solvent was removed by rotary evaporator (Sati and Joshi, 2010). These extracts were enclosed in labeled vials (Bajpai and Kang, 2010).

Evaluation of antifungal potential of the plant extracts

The evaluation of the antifungal potential of solvent extracts was done using food poisoning technique (Nene and Thapilyal, 2000; Monteiro et al., 2013). Each solvent extracts were amended with autoclaved PDA media and then poured into sterilized 90-mm petri plates. Disk of Foc (6 mm) was placed aseptically in the center of each poisoned petri plate. Plates having PDA with only 5% solvent in it, served as the control. Each treatment comprised of five replicates and plates were incubated (25±2 ˚C). Radial mycelia growth (RMG) of Foc was recorded from second day after inoculation till that day on which plates of control treatment were completely filled with mycelial growth of Foc. For each treatment, percent inhibition was calculated by using formula:

$$\text{Percent Inhibition} = \frac{\text{RMG of Control} - \text{RMG of Treatment}}{\text{RMG of Control} \times 100} \quad \text{(Eq.1)}$$

The in vitro assay and pot experiment of selected solvent extracts

Selected solvent extracts were further investigated in the *in vitro* assay using 50% concentration to determine the most effective botanical. Only 5% solvents amended with PDA, served as a solvent control. The PDA plates having only Foc disk, without any solvent and extract, served as positive control whereas the Propiconazole fungicide (Tilt) served as negative control (100 µg/mL). Assay was performed in the same manner as described earlier and percent inhibition of each treatment was calculated.

For pot experiment, four months old tissue cultured banana plants belonging to Dwarf Cavendish were grown in pots (20 cm x 25 cm) with peat moss and soil in 2:1 ratios were used as potting mix and freshly grown five day old Foc TR4 culture was used for inoculation. The inoculation of Foc was done using soil impregnation with spore suspension at 10^6 conc. (Huang et al., 2012). Four type of treatments were used in the pot assay *viz* positive control, solvent controls, negative control and solvent extracts

1) Simple control (positive control): Banana plants without any treatment.

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2) Solvent controls: n-hexane, ethanol and methanol (5% conc.) without any plant extract.
3) Fungicide treatment (negative control): Propiconazole (Tilt=100 µg/mL) was used as fungicide.
4) Botanical treatments: 50% conc. of selected solvent extract.

The banana plant roots were first dipped in the treatment (botanicals/ 5% respective solvents/ fungicide) for 35 minutes and were then sown in the potting mixture of the pots already impregnated with fungal spore suspension. Three replications for each treatment were used. Scale of external symptoms (Vicente et al., 2014) for Fusarium wilt was used for disease evaluation.

For each treatment, DSI was calculated (Huang et al., 2012) using formula:

$$\text{DSI} = \frac{\sum(\text{Class} \times \text{No.of plants in that class})}{\text{Total no.of assessed plants} \times 5} \times 100 \quad \text{(Eq.2)}$$

When the clear disease symptoms appeared on the banana plants after inoculation, visual wilt symptom assessment was done after every 2 months. Plant growth parameters i.e. leaf length, pseudostem length and leaf width were also recorded on termination of pot experiment.

Statistical analysis of experimental data

Experimental results were analyzed using the Statistix (ver. 8.1.) software. The ANOVA (analysis of variance) was performed for each experiment and the results were compared through Least Significant Difference (LSD) between means at $p<0.05$.

Gas Chromatography and Mass Spectrometry (GC-MS)

Botanicals were prepared with 100 mg/mL in 100% pure ethanol and 0.45 µm membrane filter was used to filter the botanicals. GC-MS analysis was done according to Karpagasundari and Kulothungan (2014) methodology with slight modifications. The GC-MS analysis of the most effective botanicals was executed using Shimadzu (GCMS-QP2010 Ultra) comprising an auto sampler (AOC-20i) and gas chromatograph that is interfaced to mass spectrometer (MS) instrument employed with as following specifications:

Column	DB 5 Ms (30 m × 0.25 mm × 0.25 µm)
Injection volume	3 µL (10:1split ratio)
Ion-source temperature	200ºC
Carrier gas	Helium (99.99%) at the constant flow of 1.73 mL/minute
Injector temperature	260ºC
Oven temperature	40°C (constant temperature for two minutes), with an increase of 8°C/minutes, to 150°C (constant temperature for two minutes), then 8°C/minutes to 250°C (constant temperature for two minutes), ending with a 20 minutes isothermal at 280°C
GC running time	56 minutes
Mass spectra	Taken at 70 eV, fragments from 10-1000 Da and a scan interval of 0.5 seconds

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TurboMass (Ver 5.2.0) Software was adopted to handle chromatograms and mass spectra. Database of NIST (National Institute Standard and Technology) was used to conduct interpretation on GCMS and relative peak area percentages of each component were calculated by comparing its average peak area to total areas.

Results

Screening of gymnosperm solvent extracts

Twelve gymnosperm solvent extracts recorded percent inhibition that was approximately ≥60% including 6 n-hexane extracts, 4 ethanol extracts and 2 methanol extracts (*Table 1*). Statistical analysis of solvent extract at 5 and 10% concentrations presented differences in percent inhibition values among treatments ($p < 0.05$, *Table S1*). Most of the plant solvent extracts significantly inhibited mycelial growth of Foc compared to the control treatments ($p < 0.05$, *Table S2*). The *P. wallachiana* methanol leaf extract (66.67%) and *P. wallachiana* n-hexane cone extract (66.66%) recorded highest inhibition against Foc. The n-hexane cone extracts from *Cedrus deodara* (66.07%), *P. smithiana* (66.07%), *P. roxburghii* (62.97%), *T. orientalis* (60.59%) also recorded higher inhibition against Foc. Moreover, methanol cone extract of *P. smithiana* (65.47%), ethanol leaf (62.38%) and cone (65.83%) extracts from *T. orientalis*, *P. wallachiana* ethanol leaf extract (60.12%) and *T. orientalis* n-hexane leaf extract (59.64%) also recorded higher percent inhibition values.

Table 1. *Percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents (Data presented as Mean±SE)*

Treatments	n-Hexane (extracts)		Ethanol (extracts)		Methanol (extracts)	
	5%	10%	5%	10%	5%	10%
Control	0^{n}	0^{n}	0^{n}	0^{n}	0^{n}	0^{n}
***C. sempervirens* leaf**	27.498 ± 0.98^{def}	33.69 ± 1.07^{ab}	23.45 ± 1.10^{ghi}	39.40 ± 1.86^{WX}	33.81 ± 0.74^{a}	49.64 ± 1.44^{MNO}
***C. sempervirens* cone**	32.26 ± 2.14^{abc}	51.43 ± 0.61^{KLM}	28.93 ± 0.77^{cde}	43.09 ± 2.19^{STUV}	41.19 ± 0.58^{UVWX}	54.17 ± 0.90^{IJK}
***T. orientalis* leaf**	46.90 ± 0.76^{NOPQR}	59.64 ± 0.87^{DEFG}	47.74 ± 0.35^{NOPQ}	62.38 ± 1.27^{CD}	38.45 ± 0.794^{XY}	46.08 ± 2.14^{PQRS}
***T. orientalis* cone**	55.95 ± 1.67^{HIJ}	60.59 ± 1.79^{DE}	50.24 ± 0.97^{LMN}	65.83 ± 0.35^{AB}	44.17 ± 0.51^{RSTU}	56.07 ± 1.18^{HIJ}
***C. deodara* leaf**	12.498 ± 1.22^{m}	28.81 ± 0.55^{de}	22.50 ± 1.09^{hij}	38.21 ± 0.58^{XYZ}	30.35 ± 1.23^{bcd}	39.17 ± 1.61^{WX}
***C. deodara* cone**	58.81 ± 0.47^{EFGH}	66.07 ± 1.54^{AB}	48.81 ± 0.63^{MNOP}	60.47 ± 0.55^{DE}	46.31 ± 0.44^{OPQRS}	57.74 ± 0.65^{EFGH}
***P. wallachiana* leaf**	17.26 ± 0.75^{l}	25.83 ± 4.58^{efgh}	49.17 ± 0.44^{MNOP}	60.12 ± 0.73^{DEF}	53.34 ± 0.31^{JKL}	66.67 ± 0.53^{A}
***P. wallachiana* cone**	56.31 ± 1.17^{GHIJ}	66.66 ± 1.25^{A}	18.81 ± 1.49^{kl}	34.76 ± 1.56^{a}	33.93 ± 0.33^{a}	44.64 ± 0.86^{QRST}
***P. smithiana* leaf**	26.19 ± 1.42^{efg}	34.40 ± 0.87^{a}	20.95 ± 0.98^{ijk}	35.24 ± 1.10^{YZa}	24.28 ± 2.192^{fghi}	30.35 ± 1.38^{bcd}
***P. smithiana* cone**	56.55 ± 0.94^{GHIJ}	66.07 ± 2.32^{AB}	42.02 ± 0.97^{TUVW}	57.74 ± 1.11^{EFGH}	51.78 ± 0.42^{KLM}	65.47 ± 0.37^{ABC}
***P. roxburghii* leaf**	42.26 ± 0.997^{TUVW}	51.78 ± 1.06^{KLM}	26.19 ± 0.90^{efg}	34.88 ± 0.81^{Za}	19.76 ± 0.87^{jkl}	40.47 ± 0.33^{VWX}
***P. roxburghii* cone**	52.14 ± 1.80^{KLM}	62.97 ± 0.93^{BCD}	1.55 ± 1.12^{n}	17.02 ± 1.36^{l}	46.31 ± 0.87^{OPQRS}	56.90 ± 1.04^{FGHI}

Mean values of treatments indicated by capital letter superscripts are significantly different from those of small letter superscripts and higher mean values are indicated by capital letter superscripts i.e. A, B,….Z while lower mean value are indicated by small letters i.e. a, b,….z. Values having same capital letters or small letters superscript do no differ statistically and the common letters (either capital or small letters) sharing between the treatments indicate non-significant difference (LSD = 3.38)

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The in vitro assay and pot experiment of selected solvent extracts

a) In vitro assay

Selected solvent extracts recorded significant mycelial growth inhibition as compared to the positive control and solvent controls ($p < 0.05$, *Table S3*). The fungicide control (propiconazole) and ethanol extact of *P. wallachiana* leaf recorded comparable mycelial inhibition. The *P. wallachiana* ethanol leaf extract (Pw_{EL}=99.3%) followed by ethanol leaf extract of *T. orientalis* (To_{EL}=85.9%) were found best treatments against Foc mycelial growth using 50% conc. *P. wallachiana* methanol leaf extract (Pw_{ML}=82.8%), *P. smithiana* methanol cone extract (Ps_{MC}=77.4%), *P. wallachiana* n-hexane cone extract (Pw_{HC}=75.9%) and *T. orientalis* ethanol cone extract (To_{EC}=74.6%) also recorded significant inhibitory activity against Foc (*Table 2*). Only Pw_{EL} treatment was close to the inhibitory activity of propiconazole fungicide while rest of the treatments had lower inhibition values against Foc as compared to the fungicide treatment.

Table 2. *Percent inhibition of Foc mycelia growth using 50% concentrations of the selected solvent extracts and growth parameter values and severity scoring recorded at the end of greenhouse assay (Data presented as Mean±SE)*

Treatment	Percent Inhibition (LSD = 4.28)	Length of Pseudostem in cm (LSD = 3.6)	Leaf length in cm (LSD = 4.4)	Leaf width in cm (LSD = 1.9)	Severity scores (LSD = 0.5)
Positive control	0^{I}	27.69 ± 1.2^{I}	23.83 ± 1.4^{F}	9.91 ± 0.3^{F}	5 ± 0.0^{A}
Hexane control	0^{I}	27.18 ± 1.1^{I}	26.42 ± 1.1^{EF}	12.45 ± 0.5^{E}	5 ± 0.0^{A}
Ethanol control	0^{I}	29.21 ± 0.7^{HI}	27.69 ± 0.39^{DEF}	13.12 ± 0.1^{DE}	5 ± 0.0^{A}
Methanol control	0^{I}	29.72 ± 0.9^{GHI}	27.69 ± 0.9^{DEF}	12.78 ± 0.4^{DE}	5 ± 0.0^{A}
Fungicide control	100 ± 0.0^{A}	33.02 ± 0.7^{EFG}	35.14 ± 1.8^{AB}	16.00 ± 1.0^{BC}	3 ± 0.0^{EF}
Hexane cone extract of *P. wallachiana* (Pw_{HC})	75.9 ± 1.4^{C}	37.42 ± 0.6^{BCD}	31.33 ± 0.4^{BCD}	14.65 ± 0.4^{CD}	3 ± 0.0^{EF}
Methanol leaf extract of *P. wallachiana* (Pw_{ML})	82.8 ± 1.2^{B}	39.79 ± 0.4^{ABC}	34.04 ± 1.2^{ABC}	16.17 ± 0.4^{BC}	3 ± 0.0^{EF}
Hexane cone extract of *P. smithiana* (Ps_{HC})	55.9 ± 1.2^{H}	38.9 ± 1.1^{ABCD}	33.44 ± 1.8^{BC}	16.26 ± 0.8^{ABC}	3.67 ± 0.3^{CD}
Hexane cone extract of *C. Deodara* (Cd_{HC})	66.7 ± 0.7^{EF}	39.37 ± 0.0^{ABC}	33.44 ± 1.1^{BC}	15.66 ± 0.5^{BC}	3 ± 0.0^{EF}
Ethanol cone extract of *T. orientalis* (To_{EC})	74.6 ± 1.9^{CD}	38.35 ± 1.0^{ABCD}	32.60 ± 1.1^{BC}	16.34 ± 0.7^{ABC}	3 ± 0.0^{EF}
Methanol cone extract of *P. Smithiana* (Ps_{MC})	77.4 ± 0.9^{C}	31.37 ± 3.9^{GH}	29.63 ± 3.7^{CDE}	14.73 ± 1.6^{CD}	3 ± 0.0^{EF}
Hexane cone extract of *P. roxburghi* (Pr_{HC})	70.8 ± 0.8^{DE}	38.10 ± 0.0^{ABCD}	34.63 ± 1.4^{AB}	17.36 ± 0.5^{AB}	3.67 ± 0.3^{CD}
Ethanol leaf extract of *T. orientalis* (To_{EL})	85.9 ± 0.8^{B}	40.22 ± 0.8^{AB}	33.27 ± 0.7^{BC}	16.00 ± 0.4^{BC}	2.67 ± 0.3^{FG}
Hexane cone extract of *T. orientalis* (To_{HC})	63.2 ± 0.9^{FG}	$31.75\pm0.7F^{GH}$	31.33 ± 2.2^{BCD}	15.15 ± 0.4^{C}	4 ± 0.0^{BC}
Ethanol cone extract of *C. Deodara* (Cd_{EC})	62.9 ± 1.9^{FG}	35.31 ± 0.9^{DEF}	31.50 ± 1.7^{BCD}	15.15 ± 0.7^{C}	3.33 ± 0.3^{DE}
Ethanol leaf extract of *P. wallachiana* (Pw_{EL})	99.3 ± 0.5^{A}	41.66 ± 1.2^{A}	38.27 ± 0.7^{A}	18.20 ± 0.8^{A}	2.33 ± 0.3^{G}
Hexane leaf extract of *T. orientalis* (To_{HL})	59.9 ± 4.8^{GH}	36.41 ± 1.4^{CDE}	30.06 ± 1.1^{CDE}	15.15 ± 0.7^{C}	4.33 ± 0.3^{B}

Values with same superscript letters within an individual column do no differ statistically. Common letter sharing indicate non-significant difference between the treatments

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b) Pot experiment

Banana plants in control treatments recorded visual symptoms of disease earlier compared to the majority of the treatments of solvent extract. Except fungicide control, disease severity index (DSI) of the other controls were higher compared to extract treatments. At the end of experiment, all the controls excluding fungicide control had 100% DSI and highest (5) severity scores. Minimum DSI was recorded for ethanol leaf extract of *P. wallachiana* (Pw_{EL}) i.e. 46.6%, followed by ethanol leaf extract of *T. orientalis* (To_{EL}) i.e. 53.3% DSI (*Fig. 1*). Statistical analysis presented differences in the values of severity scores ($p < 0.05$, *Table S4*), pseudostem length ($p < 0.05$, *Table S5*), leaf width ($p < 0.05$, *Table S6*) and leaf length ($p < 0.05$, *Table S7*) among treatments. Lowest severity scoring (2.33) and highest pseudostem length (41.66 cm), leaf width (18.2 cm) and leaf length (38.27 cm) had also been recorded for Pw_{EL} (*Table 2*).

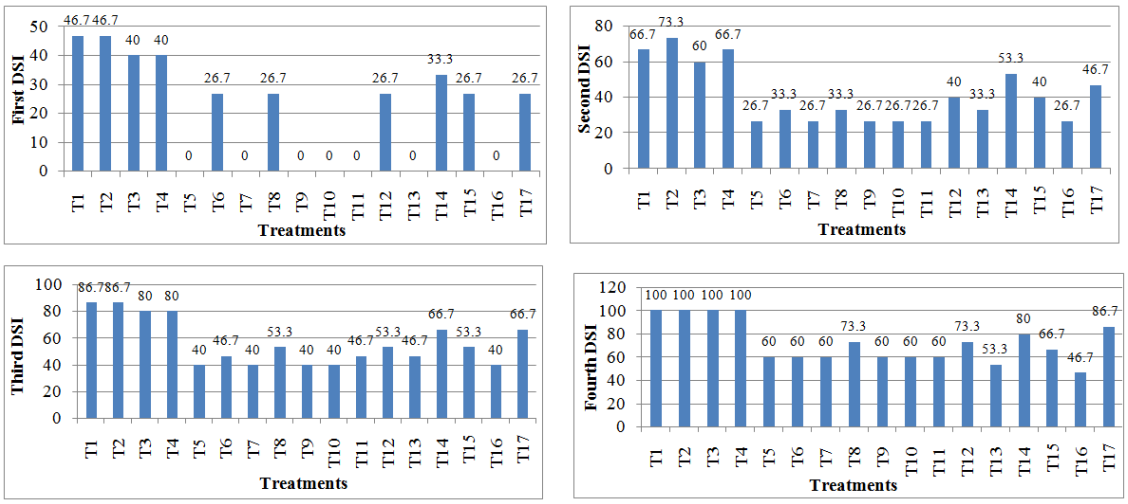

Figure 1. *Progression in the Disease severity index (DSI) of different root dipping treatments, including positive control (T_1); solvent controls (T_2-T_4); negative control (T_5) and solvent extract treatments (T_6-T_{17}), recorded during pot experiment. (T_1: Positive control, T_2: n-Hexane control, T_3: Ethanol control, T_4: Methanol control, T_5: Negative (Fungicide) control, T_6: n-Hexane cone extract of P. wallachiana, T_7: Methanol leaf extract of P. wallachiana, T_8: n-Hexane cone extract of P. smithiana, T_9: n-Hexane cone extract of C. deodara, T_{10}: Ethanol cone extract of T. orientalis, T_{11}: Methanol cone extract of P. smithiana, T_{12}: n-Hexane cone extract of P. roxburghi, T_{13}: Ethanol leaf extract of T. orientalis, T_{14}: n-Hexane cone extract of T. orientalis, T_{15}: Ethanol cone extract of C. deodara, T_{16}: Ethanol leaf extract of P. wallachiana, T_{17}: n-Hexane leaf extract of T. orientalis)*

GC-MS analysis for Pw_{EL} and To_{EL}

The total ion chromatogram (TIC) of Pw_{EL} detected seven compounds having retention time ranging between 19.1 to 41.6 minutes (*Fig. 2*). The delta-Cadinene, Vitamin C, cis-9-Hexadecenal, Oleic acid, 1-Naphthalenecarboxylic acid, 1- Naphthalenepentanoic acid and Hexatriacontyl trifluoroacetate were present in Pw_{EL}. The 1-Naphthalenecarboxylic acid, Vitamin C and Hexatriacontyl trifluoroacetate were detected with high peak area percent (*Table 3*). Total ion chromatogram of To_{EL} also detected seven compounds having retention time ranging between 21.06 to 36.2 minutes (*Fig. 2*). The Cedrol, cis-9-Hexadecenal, Podocarp-7-en, Pimaric acid,

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1-Phenanthrenecarboxylic acid, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid were detected in To_{EL}. The 1-Naphthalenecarboxylic acid (Hardwickiic acid), Cedrol and 1-Naphthalenepentanoic acid were found with high percentage of peak area (*Table 3*). The 1-Naphthalenepentanoic acid, 1-Naphthalenecarboxylic acid and cis-9-Hexadecenal were noticed in the two extracts (*Fig. 3*).

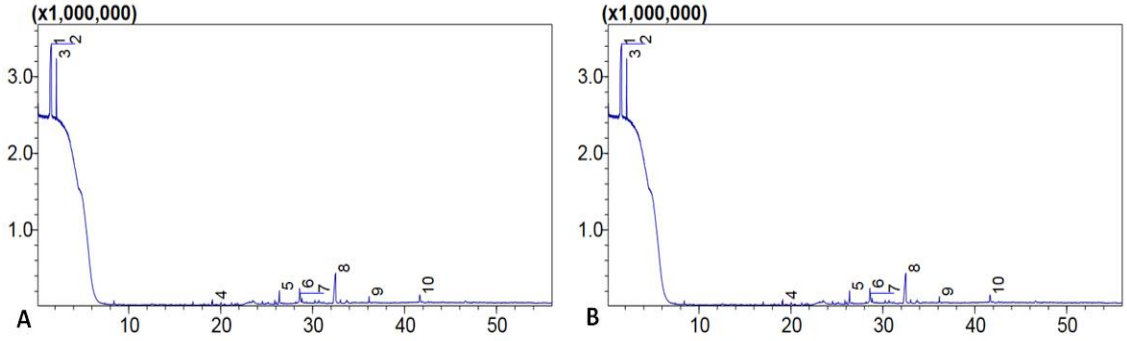

Figure 2. *Total ion chromatogram of Pw_{EL} (A) and To_{EL} (B) obtained through GCMS analysis*

Table 3. *Compounds detected in Pw_{EL} and To_{EL} with GC-MS analysis*

Peak	Compounds in Pw_{EL}				Compounds in To_{EL}			
	R_T	**Area%**	**Name/ Formula/ Molecular weight**	**Chemical group**	**R_T**	**Area%**	**Name/ Formula/ Molecular weight**	**Chemical group**
1	1.55	41.8	Ethanol/ C_2H_6O/ 46	Solvent	1.59	55.0	Ethanol/ C_2H_6O/ 46	Solvent
2	1.59	11.4	Ethanol/ C_2H_6O/ 46	Solvent	2.14	11.0	Chloroform/ $CHCl_3$/ 118	Solvent
3	2.14	10.3	Chloroform/ $CHCl_3$/ 118	Solvent	21.06	3.9	Cedrol/ $C_{15}H_{26}O$/ 222	Sesquiterpene alcohol
4	19.11	1.0	delta-Cadinene/ $C_{15}H_{24}$/ 204	Cadinene family of sesquiterpines	28.60	2.9	cis-9-Hexadecenal/ $C_{16}H_{30}O$/ 238	Palmitole aldehyde
5	26.39	3.1	Vitamin C / $C_{38}H_{68}O_8$/ 652	Ester	28.66	1.1	Podocarp-7-en-3-one, 13.beta.-methyl-1/ $C_{20}H_{30}O$/ 286	Diterpenoid/ meroterpene natural phenol
6	28.59	3.7	cis-9-Hexadecenal/ $C_{16}H_{30}O$/ 238	Palmitole Aldehyde	30.61	1.7	Podocarp-7-en-3-one, 13.beta.-methyl-1/ $C_{20}H_{30}O$/ 286	Diterpenoid/ meroterpene natural phenol
7	28.64	1.4	Oleic acid/ $C_{18}H_{34}O_2$/ 282	Monounsaturated (omega-9) fatty acid	31.26	2.3	Pimaric acid/ $C_{20}H_{30}O_2$/ 302	Carboxylic acid from the resin acid group
8	2.51	22.6	1-Naphthalenecarboxylic acid / C20H28O3/ 316	Clerodane diterpenoid	32.00	3.3	1-Phenanthrenecarboxylic acid / $C_{21}H_{32}O_2$/ 316	Methyl ester/ Neoabietic acid
9	36.14	1.5	1-Naphthalenepentanoic acid/ $C_{22}H_{36}O_4$/ 364	Methyl ester	32.47	8.9	1-Naphthalenecarboxylic acid /$C_{20}H_{28}O_3$/ 316	Clerodane diterpenoid
10	41.64	3.1	Hexatriacontyl trifluoroacetate/ $C_{38}H_{73}F_3O_2$/ 618	Alcohol acetate	36.21	9.8	1-Naphthalenepentanoic acid/ $C_{22}H_{36}O_4$/ 364	Methyl ester

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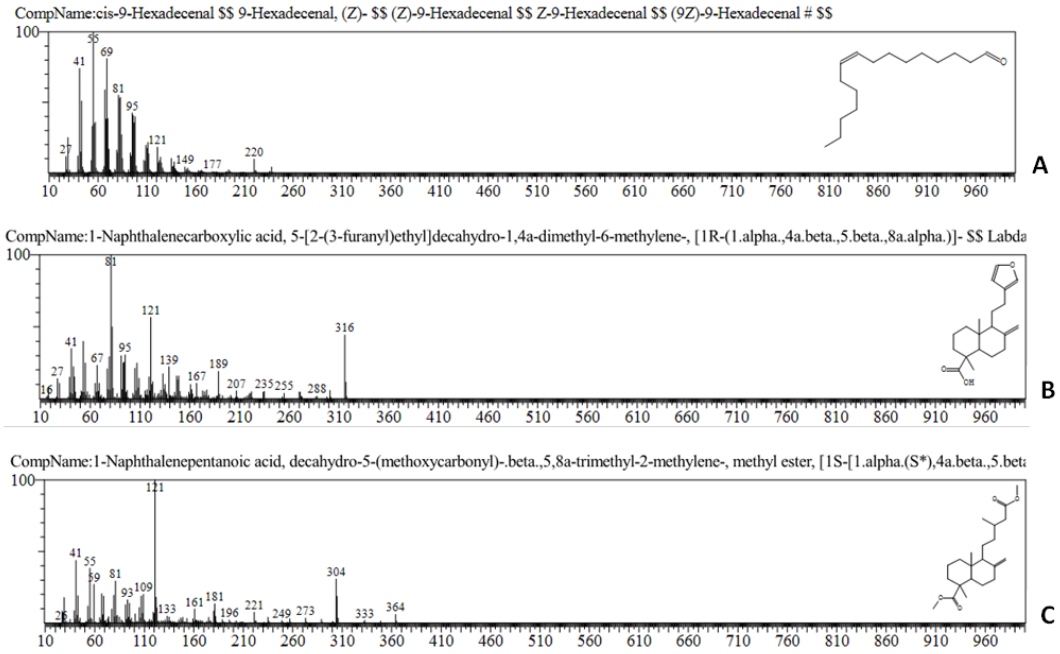

Figure 3. *Standard molecular spectra of cis-9-Hexadecenal (A), 1-Naphthalenecarboxylic acid (B) and 1-Naphthalenepentanoic acid (C) detected in* Pw_{EL} *and* To_{EL}

Discussion

Modern science is using huge array of plants because of their antimicrobial traits, which are attributed to compounds synthesized in the secondary metabolism of the plant (Nascimento, 2000). Terpenoids, tannins, ligans, steroids, alkaloids, phenols, glycosides, and sugar derivatives are noted as secondary metabolites in the gymnosperms (Harborne and Baxter, 2001). Plant secondary metabolites structure has been optimized during evolutionary process so that they can interfere with microbial molecular targets thus acting as a defense mechanism (Wink et al., 2012). Plant extracts disrupts the normal cell functioning of microbes, thus affecting important steps in the pathogenic process (Gupta and Birdi, 2017). Botanicals comprises such phytocompounds that cause morphological alterations in fungal hyphae which results to shriveling of hyphae, protoplast leakage and vacuolations coagulation (Soylu et al., 2006). The modes of action of phytoproducts on cells of fungi are thought to be membrane rupture in cytoplasm, granulation of cytoplasm, inhibition of extracellular and intracellular synthesis of enzymes (Cowan, 1999). Result of preliminary *in vitro* screening for gymnosperm solvent extracts recorded significant inhibition of Foc compared to the control treatments that clearly indicates antifungal efficacy of the tested gymnosperm plants. Joshi and Sati (2012) described antimicrobial efficacy of botanicals from many plant species belonging to various families of gymnosperms and current study is in accordance with their findings. Although, antimicrobial potential of gymnosperm botanicals has been reported against various tested pathogens but their antifungal efficacy against Foc is never been tested. This study first time reports the efficacy of solvent extracts of selected gymnosprem plants against Foc. It was noted that some extracts had similar inhibition activity with all three solvent types while some extracts recorded different inhibition potential using three solvents. This might be because of the variable content of phytochemicals in each plant extract and

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because of different extraction potentials of each solvent. Khoddami et al. (2013) described that type of plant/plant part and extraction solvent are reason for the variation of phytochemical formation in various extracts. Altemimi et al. (2017) noticed solvent effects on the phytochemical constituent profiles and antioxidant activities of different plant extracts.

Twelve solvent extracts selected through antifungal screening and further evaluated in the *in vitro* assay and green house assay, reported efficacy of Pw_{EL} comparable to fungicide (propiconazole) treatment. Highest mycelial inhibition against Foc indicated that Pw_{EL} was the most efficient solvent extract, suggesting presence of comparatively higher amount of effective phytochemicals in their composition, which effectively restricted and inhibited Foc mycelial growth. Sharma et al. (2018) recorded insecticidal and antimicrobial activities of *P. wallachiana* leaf extracts. Sharma et al. (2015) also noted antioxidant and antibacterial activities of alcoholic extracts of *P. wallachiana* leaf. Highest values in growth parameters and minimum DSI were recorded for Pw_{EL} followed by To_{EL} compared to the control treatments in green house assay. Huang et al. (2012) and Gopi and Thangavelu (2014) noted that botanical treatments effectively suppressed Fusarium wilt in green house experiments.

GCMS analysis of Pw_{EL} and To_{EL} detected seven compounds in each extract. Some reported compounds have well-known antimicrobial efficacies i.e. Oleic acid, 1-Naphthalenepentanoic acid, cis-9-Hexadecenal, delta-Cadinene, 1-Naphthalenecarboxylic acid (Hardwickiic acid), Cedrol and 1-Phenanthrene carboxylic acid. Dar et al. (2012) reported delta-Cadiene as one of the chemical constituent detected from the essential oil of *P. wallachiana* needle. Joshi et al. (2016) described thirty-eight compounds in the *P. wallachiana* methanolic leaf extracts including Vitamin C. Anburaj et al. (2016) reported Vitamin C as antioxidant, immunomodulator and anticancer. Jang et al. (2016) noted antimicrobial activity from *Eleutherococcus senticosus* essential oil containing delta-Cadinene as one of its compositional compounds. The cis-9-Hexadecenal has been reported for its antimicrobial activity (Mujeeb et al., 2014). Awa et al. (2012) noticed antibacterial activity of Oleic acid. McChesney et al. (1991) described antimicrobial potential of 1-Naphthalenecarboxylic acid (Hardwickiic acid) isolated from the *Croton sonderianus*. Similarly, Kuete et al. (2007) also revealed antimicrobial potential of compounds (including Hardwickiic acid) extracted from *Irvingia gabonensis* stem bark.

Khubeiz et al. (2016) and Moawad and Amin (2019) noted sesquiterpenoids and monoterpenes as chemical components from leaf essential oil of *T. orientalis* containing Cedrol as one of the major component recording significant antibacterial activity. Madhumitha et al. (2012) described insecticidal efficacy of aqueous extract fruit peel of *Annona squamosa* against parasites (blood feeding). Its constitutional chemical compounds included Podocarp-7-en. Naikwadi et al. (2017) revealed antibacterial potential of *Vetiveria zizanioides* root extracts containing 1-Phenanthrene carboxylic acid, Phenanthrene carboxylic acid and Naphthalene pentanoic acid as major bioactive constituents. Manimegalai et al. (2011) isolated compounds from *T. orientalis* bark that were antibacterial and separation by TLC and GCMS disclosed presence of Phenanthrene carboxylic acid in it. Ali et al. (2011) reported anti-inflammatory and antibacterial potency of Pimaric acid.

Compounds that were detected through GCMS in Pw_{EL} and To_{EL} might be the probable phytoconstituents for Foc control in the *in vitro* and pot assays. These phytochemicals either through stimulating host's defence response by activating PR-proteins or directly

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toxifying Foc, inhibited mycelial amelioration in the pot and *in vitro* experiments respectively. Zhang et al. (2013) described that volatiles derived from plant source displayed higher inhibition potential on Foc and the compound i.e. 2-methyl-2 pentenal, completely inhibited mycelial growth of Foc. Gopi and Thangavelu (2014) reported lipid compound in botanical treatment as effective constituent that significantly suppressed Panama disease under greenhouse assay. Guimaraes et al. (2011) and Siripornvisal (2010) also noted antifungal potential of volatile compounds against Foc inhibiting spore germination and mycelial growth. Three compounds i.e. 1-Naphthalenepentanoic acid, 1-Naphthalenecarboxylic acid and cis-9-Hexadecenal, were revealed in both extracts that might be the potential active components of Pw_{EL} and To_{EL}. According to our knowledge, cis-9-Hexadecenal, 1-Naphthalenecarboxylic acid (Hardwickiic acid), Oleic acid, Hexatriacontyl trifluoroacetate and 1-Naphthalenepentanoic acid are noticed in Pw_{EL}, for the first time, through current study. Moreover, Podocarp-7-en, cis-9-Hexadecenal, 1-Phenanthrenecarboxylic acid, Pimaric acid, 1-Naphthalenepentanoic acid and 1-Naphthalenecarboxylic acid are also reported first time in To_{EL} as most of the phytochemical studies on *Thuja orientalis* are focused on its essential oil which only reports cedrol presence.

Conclusion

A baseline study that for the first time addresses Fusarium wilt dilemma using botanicals and reports antifungal efficacy of selected gymnosperms against Foc. Even though variability was found in the inhibitory activity of different gymnosperm solvent extracts, However, few solvent extracts recorded good efficacy against Foc both in the *in vitro* and pot experiments. The ethanolic leaf extract of *P. wallachiana* (Pw_{EL}) and the ethanolic leaf extract of *T. orientalis* (To_{EL}) were evidenced through GCMS to have bountiful sources of such valuable phytochemicals that can replace conventional chemicals and fungicides that are used for the management of Panama wilt disease. It is therefore, strongly recommended that the efficient gymnosperm extract should be further explored through spectrophotometric and chromatographic analysis. Moreover, compound detected through GCMS should be individually evaluated against Foc using *in vitro* and green house experiments.

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APPENDIX

Supplementary Information

Table S1. *Analysis of variance (factorial 3-way) applied for percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents ($p < 0.05$)*

Source	DF	SS	MS	F	P
Replicate	4	10	2.4		
Treatment	12	84953	7079.5	956.93	0.0000
Solvent	2	3019	1509.4	204.03	0.0000
Conc	1	12178	12178.4	1646.15	0.0000
Treatment*Solvent	24	32780	1365.8	184.62	0.0000
Treatment*Conc	12	1235	102.9	13.91	0.0000
Solvent*Conc	2	200	99.8	13.48	0.0000
Treatment*Solvent*Conc	24	831	34.6	4.68	0.0000
Error	308	2279	7.4		
Total	389	137485			

Grand Mean 39.656, CV 6.86

Table S2. *Pairwise comparisons test (Treatment*Solvent*Concentration) of percent inhibition of Foc mycelia growth recorded for 5% and 10% of gymnosperm extracts prepared in n-hexane, ethanol and methanol solvents*

Treatment	Solvent	Conc	Mean	Homogeneous Groups
8	3	2	66.666	A
9	1	2	66.664	A
7	1	2	66.074	AB
11	1	2	66.070	AB
5	2	2	65.832	AB
11	3	2	65.474	ABC
13	1	2	62.974	BCD
4	2	2	62.384	CD
5	1	2	60.592	DE
7	2	2	60.474	DE
8	2	2	60.116	DEF
4	1	2	59.642	DEFG
7	1	1	58.808	EFGH
11	2	2	57.738	EFGH
7	3	2	57.736	EFGH
13	3	2	56.902	FGHI
11	1	1	56.546	GHIJ
9	1	1	56.306	GHIJ

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Treatment	Solvent	Conc	Mean	Homogeneous Groups
5	3	2	56.068	HIJ
5	1	1	55.952	HIJ
3	3	2	54.172	IJK
8	3	1	53.338	JKL
13	1	1	52.138	KLM
11	3	1	51.784	KLM
12	1	2	51.780	KLM
3	1	2	51.428	KLM
5	2	1	50.238	LMN
2	3	2	49.644	MNO
8	2	1	49.166	MNOP
7	2	1	48.810	MNOP
4	2	1	47.736	NOPQ
4	1	1	46.902	NOPQR
7	3	1	46.308	OPQRS
13	3	1	46.308	OPQRS
4	3	2	46.078	PQRS
9	3	2	44.642	QRST
5	3	1	44.166	RSTU
3	2	2	43.094	STUV
12	1	1	42.260	TUVW
11	2	1	42.020	TUVW
3	3	1	41.186	UVWX
12	3	2	40.474	VWX
2	2	2	39.404	WX
6	3	2	39.168	WX
4	3	1	38.448	XY
6	2	2	38.212	XYZ
10	2	2	35.240	YZa
12	2	2	34.880	Za
9	2	2	34.764	a
10	1	2	34.404	a
9	3	1	33.928	a
2	3	1	33.808	a
2	1	2	33.692	ab
3	1	1	32.260	abc
6	3	1	30.354	bcd
10	3	2	30.354	bcd
3	2	1	28.926	cde
6	1	2	28.810	de
2	1	1	27.498	def
10	1	1	26.188	efg
12	2	1	26.188	efg
8	1	2	25.834	efgh
10	3	1	24.284	fghi
2	2	1	23.452	ghi
6	2	1	22.498	hij
10	2	1	20.952	ijk
12	3	1	19.762	jkl
9	2	1	18.808	kl
8	1	1	17.260	l
13	2	2	17.022	l
6	1	1	12.498	m
13	2	1	1.5480	n
1	1	1	0.0000	n
1	1	2	0.0000	n
1	2	1	0.0000	n
1	2	2	0.0000	n
1	3	1	0.0000	n
1	3	2	0.0000	n

Alpha 0.05 Standard Error for Comparison 1.7202, Critical T Value 1.968, Critical Value for Comparison 3.3849, Error term used: Replicate*TREATMENT*Solvent*Conc., 308 DF, there are 40 groups (A, B, etc.) in which the means are not significantly different from one another

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Table S3. *Completely randomized analysis of variance for percent inhibition of Foc mycelia growth using 50% concentrations of the selected solvent extracts (p< 0.05)*

Source	DF	SS	MS	F	P
Treatment	16	97956.4	6122.28	532	0.0000
Error	68	781.9	11.50		
Total	84	98738.4			

Grand Mean 57.387, CV 5.91

Table S4. *Completely randomized analysis of variance for severity scoring of banana plants treated with different solvent extracts in green house experiment (p< 0.05)*

Source	DF	SS	MS	F	P
Treatment	16	39.6471	2.47794	21.1	0.0000
Error	34	4.0000	0.11765		
Total	50	43.6471			

Grand Mean 3.6471, CV 9.40

Table S5. *Completely randomized analysis of variance for measurement of banana pseudostem length measured in different treatments during greenhouse experiment (p< 0.05)*

Source	DF	SS	MS	F	P
Treatment	16	1085.16	67.8225	13.	0.0000
Error	34	167.47	4.9257		
Total	50	1252.63			

Grand Mean 35.030, CV 6.34

Table S6. *Completely randomized analysis of variance for leaf width of banana plants measured in different treatments during greenhouse experiment (p< 0.05)*

Source	DF	SS	MS	F	P
Treatment	16	192.539	12.0337	8.65	0.0000
Error	34	47.312	1.3915		
Total	50	239.850			

Grand Mean 15.006, CV 7.86

Table S7. *Completely randomized analysis of variance for leaf length of banana plants measured in different treatments during greenhouse experiment (p< 0.05)*

Source	DF	SS	MS	F	P
Treatment	16	619.498	38.7186	5.35	0.0000
Error	34	246.141	7.2394		
Total	50	865.639			

Grand Mean 31.429, CV 8.56

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