Towards The Management of Napier Head Smut; Using *In vitro* Approaches to Decipher Possible Role of Antibiosis Resistance in Selected Napier Accessions

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A B S T R A C T

A glasshouse factorial experiment in completely randomized design coupled with laboratory assays were conducted at Kenya Agricultural and Livestock Research Organization-Muguga south and National Agricultural Research Laboratories-Kabete Kenya. The study was conducted with the specific objective being to decipher the possible strategy of resistance involved in napier grass accessions' management of head smut disease through host plant resistance. The sample study entailed selected asymptomatic and molecularly screened napier grass accessions which had been acquired from ILRI germplasm bank. Laboratory assays were conducted where the purposively sampled accessions' crude extracts were used from second harvest point to determine their effect on the pathogen's lag phase *in vitro*. Crude extracts from accession 16805 and Kakamega 1 resistant variety exhibited the longest lag phases of 18.5 ($R^2 = 0.9969$) and 18.2 ($R^2 = 0.9969$) hours respectively, whereas Clone 13 a susceptible variety and blank control exhibited the shortest lag phases of 17.1 ($R^2 = 0.9977$) and 15.3 ($R^2 = 0.9965$) hours respectively. The results indicated that the strategy of resistance used by the accessions to combat the pathogen establishment seems antibiosis. However, further elucidation of the observed resistance phenomenon will be critical through biochemical methods and fluorescence microscopy to fully optimize on the accession's resistance as potential breeding candidates for superior clones towards effective management of the napier head smut disease.

Keywords: Antibiosis, Head smut, Napier grass, Assay, Antixenosis.

INTRODUCTION

Napier grass is an important fodder source in Kenya and Eastern Africa region to smallholder dairy farmers (Anitha et al., 2006, ASARECA, 2010, Omayio et al., 2014). Thus, its cultivation has been on the rise in tandem with the dairy industry's growth (Farrell et al., 2002; Orodho, 2006; ASARECA, 2010). Despite, this tremendous boost of the milk industry by the crop in terms of feed provision; in recent years it has been experiencing a serious production constraint in the form of napier head smut disease caused by *Ustilago kamerunensis* (Farrell et al., 2002; Orodho, 2006). The disease is widespread in Central Kenya region where it causes yield losses of up to 46% (Farrell et al., 2000; Orodho, 2006). Moreover, of concern has been its continual

spread to other parts of the country like the Rift-valley and lower Eastern regions of the East African country (Lukuyu et al., 2012).

Efforts have been put in place so far to mitigate this disease through exploitation of the host plant resistance exhibited by some varieties of napier grass (*Pennisetum purpureum*) developed earlier like Kakamega 1 and 2 against the pathogen. An approach which has largely been driven by its ease of implementation and effectiveness across the small holder dairy farmers in terms of cost and efficacy (Parry, 1990; Orodho, 2006). This host plant resistance is genome based and it's manifested through various strategies ranging from induced to constitutively produced factors (Parry, 1990). This then form the physiological basis of resistance which may be manifested through either structural (antixenosis) or biochemical (antibiosis) tactics of defense singly or together (Parry, 1990; Robert et al., 2003). Selection of asymptomatic napier grass accessions against head smut disease has continued to be done to increase the gene pool of resistance genes to the pathogen and avert a likely breakdown of the same by a possible evolved strain of the pathogen (NAFIS, 2012). However, the strategy of resistance used by the fodder crop has not been determined. Farrell (1998) ruled out the likely involvement of physical barriers in the resistance although his investigation did not extend to the other possible alternatives. This information is critical towards coming up with breeding goals for superior clones development and management of the disease effectively. Therefore, this study sought to establish and lay a platform on understanding of the likely alternative resistance strategy used by the accessions in combating the pathogen's establishment.

MATERIALS AND METHODS

Identification of the napier grass accessions' study sample

The glasshouse study was conducted at Kenya Agricultural and Livestock Research Organization-Muguga South, located at Kiambu County in Kenya. This was coupled with laboratory assays at the National Agricultural Research Laboratories located at KALRO-Kabete in Nairobi Kenya. Six purposively sampled ex-ILRI accessions presumed to exhibit different threshold levels of tolerance to napier head smut (table 1) selected by Omayio et al. (2014) were evaluated. The presumed tolerant accessions were purposively sampled basing on the ones exhibiting high dry matter content per neighbour joining group, with exception of 16808 which was selected due to its lowest dry matter content among the asymptomatic accessions as reported by Omayio et al. (2014). Two local napier grass variety checks (Kakamega 1 and Clone 13) were used as negative and positive checks to head smut as they have been validated as resistance and susceptible to the disease respectively to give a total of eight accessions evaluated for their extracts properties.

Table 1: Purposively sampled accessions presumed to exhibit some level of tolerance

Napier accessions	Percentage dry matter	Neighbour joining group
16808	19.88%	East Africa
16902	24.10%	Hybrid
16805	22.61%	USA 2
16785	27.85%	Southern Africa
16783	23.83%	Miscellaneous
16811	24.68%	USA 1
Kakamega 1	Negative check	Unknown
Clone 13	Positive check	Unknown

Source: Omayio et al. (2014)

Experiment one: Inoculation of the accessions at glasshouse level

This experiment was conducted to lay a platform for laboratory assays of the napeir grass extracts. A factorial experiment testing two factors namely; the asymptomatic accessions and inoculation levels (that is inoculated and uninoculated control) in a completely randomized design was set up. Napier planting materials and preparation of the innoculum was done following the procedure described by Mwendia et al. (2006). The innoculum of *Ustilago kamerunensis* ustilospores was prepared and standardized using haemocytometric techniques as per (Kinyua, 2004). The concentration target was 5×10^6 spores/ml as used previously in the screening of Kakamega 1 (Farrell, 1998). Accessions inoculation was by dipping method as described by Mwendia et al (2006) and Farrell, (1998). The inoculated canes were then planted at an angle with one third of the cane above the soil in plastic pots of between 27 cm diameter filled with potting mixture. The potting mixture comprised of non-sterile soil, gravel and cattle manure at a ratio of 4: 1: 0.75 respectively. The respective treatment combinations were replicated thrice with two canes planted per pot to give a total of six data points per accession's treatments. Therefore, fresh canes of the asymptomatic accessions purposively sampled as indicated on table I, were established along with Kakamega 1 and Clone 13 as a resistant and a susceptible check respectively. Uninoculated controls dipped in only distilled water without the ustilospores were introduced and replicated in the same way. The plants were watered once in the evenings at 6 p.m after a days interval. The accessions were harvested on week 8 (first ratoon) to allow for regrowth and then on week 16 (second ratoon) for the extracts' evaluation.

Extraction of the xylemparenchymatous and leaf extracts' crude mixture of the accessions

The extraction procedure entailed the use of a modified methodology by (Paul and Sharma, 2002; Sanjay and Ashok, 2006; Das et al., 2010; Norhafizah et al., 2012). The above extraction was conducted on the second ratoon's harvest when the disease was expected to have established. The above ground tissues from the inoculated and uninoculated control of the purposively sampled asymptomatic accessions as indicated on (table 1) and their susceptible and resistant checks Clone 13 and Kakamega 1 respectively were used. The tissues were cleaned and cut into a length of 1cm. Then 4 grams (fresh weight) of the mixture (2 grams xylem-parenchyma tissue and 2 grams leaf tissues) were then homogenized in a pre-chilled pestle and mortar or tissue homogenizer, using 10ml chilled sterilized distilled water per each 4 grams of each accession. The mixture was then soaked and shaken vigorously at 200 rpm for 15 minutes on orbital shaker at 25°C. The extract was filtered through moistened muslin cloth and then four cycles using whatman No.1 filter papers. The concentration of the sample was finally increased by centrifuging at 2000 gravity, at 4°C for 15 minutes to obtain the supernatant for each accession's extract which was stored at 4°C for use in laboratory assaying of the pathogen *Ustilago kamerunensis*.

In vitro assessment of the pathogen using the napier accessions' extracts

The effects of the respective accession's crude extracts on the lag phase of the pathogen *Ustilago kamerunensis* was tested in a modified poisoned food technique (Dhingra and Sinclair, 1985). 1 ml of each accession's extract was pour plated in 10 ml petri dishes containing sterilized oxoid malt extract agar at 121°C for 15 minutes. This media was treated with 10ml lactophenol per litre during preparation to inhibit bacterial growth. Then a 10μl volume pre-standardized pathogen spore inoculum concentration of 5×10^6 spores ml^{-1} as described by Kinyua (2004), was spot inoculated at the centre of each plate under a lamina air flow chamber (Andrea et al., 2005). The inoculations for each treatment were replicated 10 times in a completely randomized design, with a blank control containing only the pathogen culture without any pour plated crude extract. The inoculated plates were then incubated at 25°C upon sealing them using a parafilm in a dark area. The top white floccose and reverse pale cream colonies radii were measured once daily at 4 p.m up to the 7th day after incubation. Two radii, measured at right angles to one another were averaged to find the mean radius for the colony. Accessions' extracts effect on pathogen growth per day was determined by averaging the radii from the two sets of five petri plate replicates per treatment for each accession (Andrea et al., 2005).

Statistical analysis procedure

Univariate analysis of variance on the radial growth of the pathogen was performed to determine whether there were significant differences on the napier accessions' extracts effect on the pathogen's growth. Then a regression analysis of the pathogens growth basing on the averaged radii under respective napier grass accessions' extracts challenge was performed to determine the lag phase time of the pathogen's (*Ustilago kamerunensis*) growth. The point where the linear plot cut the x-axis when y = 0 was taken as the lag phase of the pathogen in a plot of radial growth in centimetres against time in days.

RESULTS AND DISCUSSION

The crude extracts' effect on the lag phase of the pathogen in vitro

Significant differences (df = 1; F= 5.69; p= 0.0183) were observed at critical level P ≤ 0.05 in the treatment of the pathogen with the extracts from the inoculated and uninoculated controls of the respective accessions as shown on the repeated measures ANOVA table 2. The lag phases were determined using the repeated measures ANOVA means and the results summarized on a table 3. Extracts from accession 16805 inoculated samples produced the longest lag phase of 18.5 (R^2 = 0.9969) hours followed by Kakamega 1 at 18.2 (R^2 = 0.9969) hours. The shortest lag phases observed stood at 17.1 (R^2 = 0.9977) hours and 15.3 (R^2 = 0.9965) hours for the Clone 13 and the blank control respectively. As observed on table 3, the extracts from the non-inoculated controls of the respective accessions exhibited a slightly shorter lag phase on the pathogen growth *in vitro* than those from their inoculated samples. This could be attributed to biochemical defenses in the napier grass accessions that arise from induced enzyme activity that catalyze the development of confrontational strategies against the *Ustilago kamerunensis*. Similar, observation has been reported in sugarcane crop; where crude fungal elicitors have induced phenylalanine ammonia lyase and peroxidase production against sugarcane head smut (*Ustilago scitaminea*) disease (Santiago et al., 2008). Moreover, the sugarcane crop attacked by this head smut (*Ustilago scitaminea*) pathogen, has been observed produce increased levels of glycoproteins with cytoagglutination properties as chemical defense (Blanca et al., 2002; Ana-Maria et al., 2005).

Further, in the pearl millet (*Pennisetum glaucum*) a very close member to napier grass; similar resistant inducting enzymes viz. peroxidase, phenylalanine ammonia lyase, polyphenol oxidase, chitinase and β-1, 3-glucanase have been observed against downy mildew infection (Niranjan et al., 2012). Furthermore, this biochemical defense phenomenon is supported by the accession 16902 selection as one of the resistant accessions by Omayio et al. (2014) which is a hybrid of *P. purpureum* and *P. glaucum* (Lowe et al., 2003). Finally, for the observed differences of the controls lag phases to the blank's could be attributed to

the constitutively produced glycoproteins that accumulate in parenchymatous cells (Ana-Maria et al., 2005) or the differences in water activity that influence growth of microbes in culture (Andrea et al., 2005).
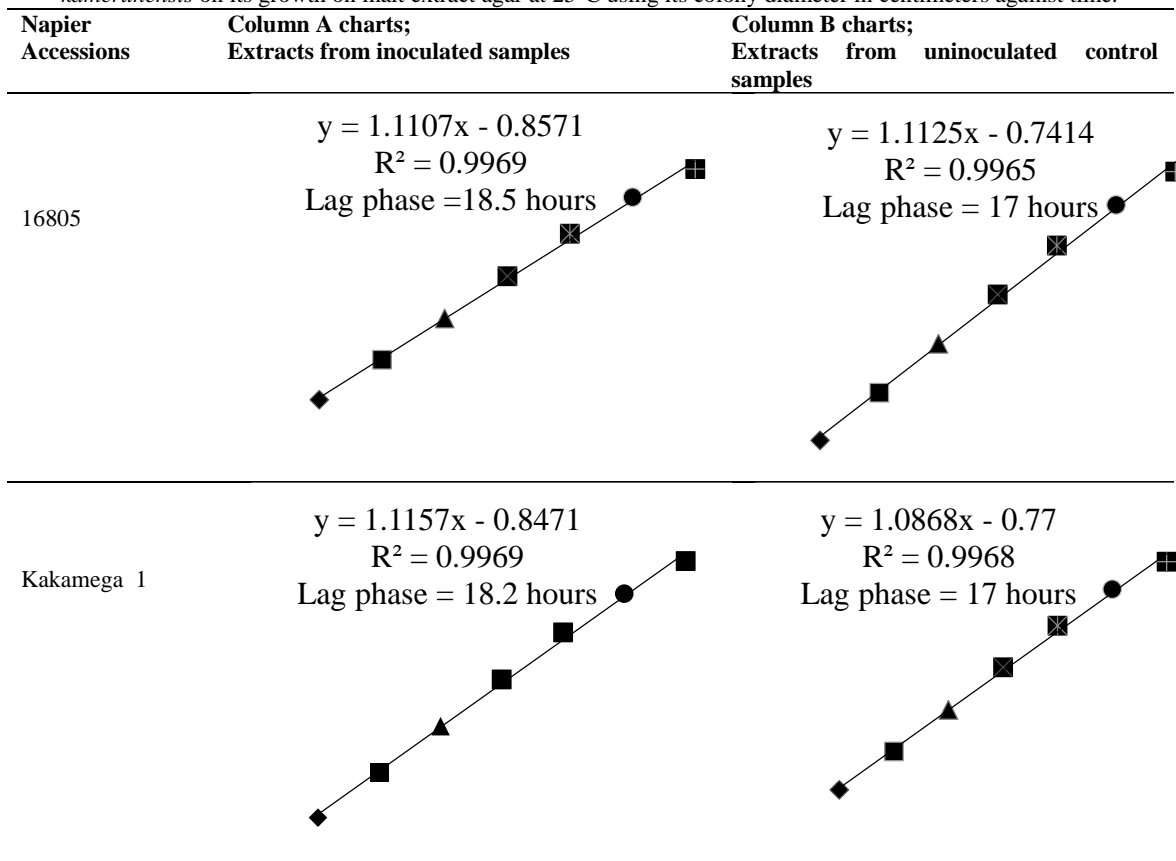
The study concludes that the napier grass accessions seem to involve biochemical factors (antibiosis form of defense) in the challenge of *Ustilago kamerunensis* establishment within the tissues of the selected asymptomatic accessions. This is basing on the observed effects of the crude extracts on the pathogen *in vitro*. However, the observed aspects need to be elucidated further; where the various biochemical resistance marker enzymes (pathogenesis related proteins) involved in such resistance viz; polyphenol oxidase, phenylalanine ammonia lyase, chitinase, β-1, 3-glucanase, lipozygenase and peroxidases need to be assayed if they are involved using biochemical techniques so as to elucidate the observed effects to pave way for more understanding of this resistance from the various pathways involved such as salicylic acid, ethylene and jasmonic acid (Pieterse et al., 2001). Also, the likely complementation of the chemical defenses by generation of internal physical barriers like callose (β - 1, 3 - glucan) deposition, hydroxylated methoxylated phenylpropane polymer and possibility of enhanced lignifications needs to be elucidated through histo-chemical analysis and fluorescence microscopy. This is because such physical barriers have been observed in *Pennisetum glaucum* against downy mildew (*Sclerospora graminicola*) which is in the same genus as napier grass.
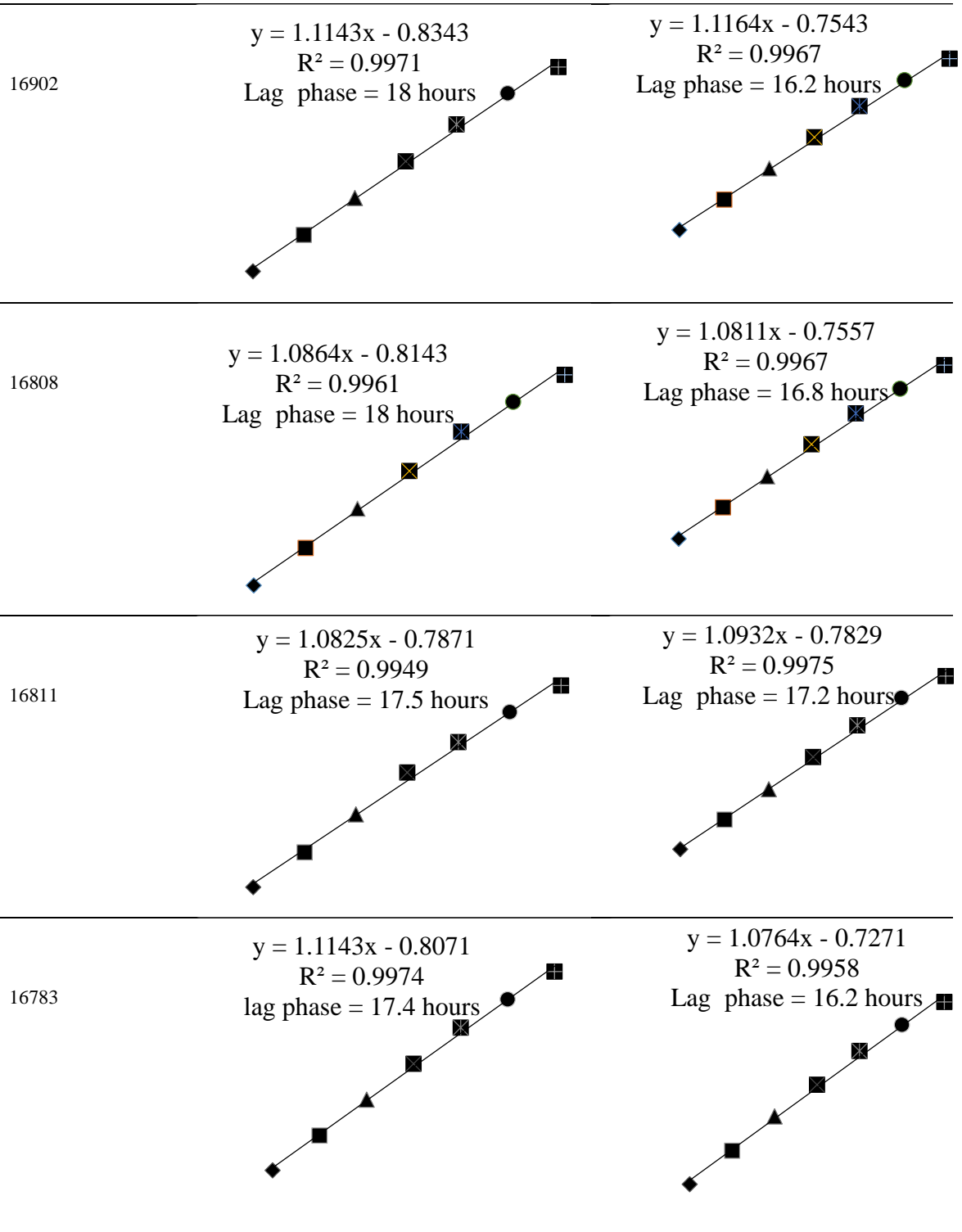
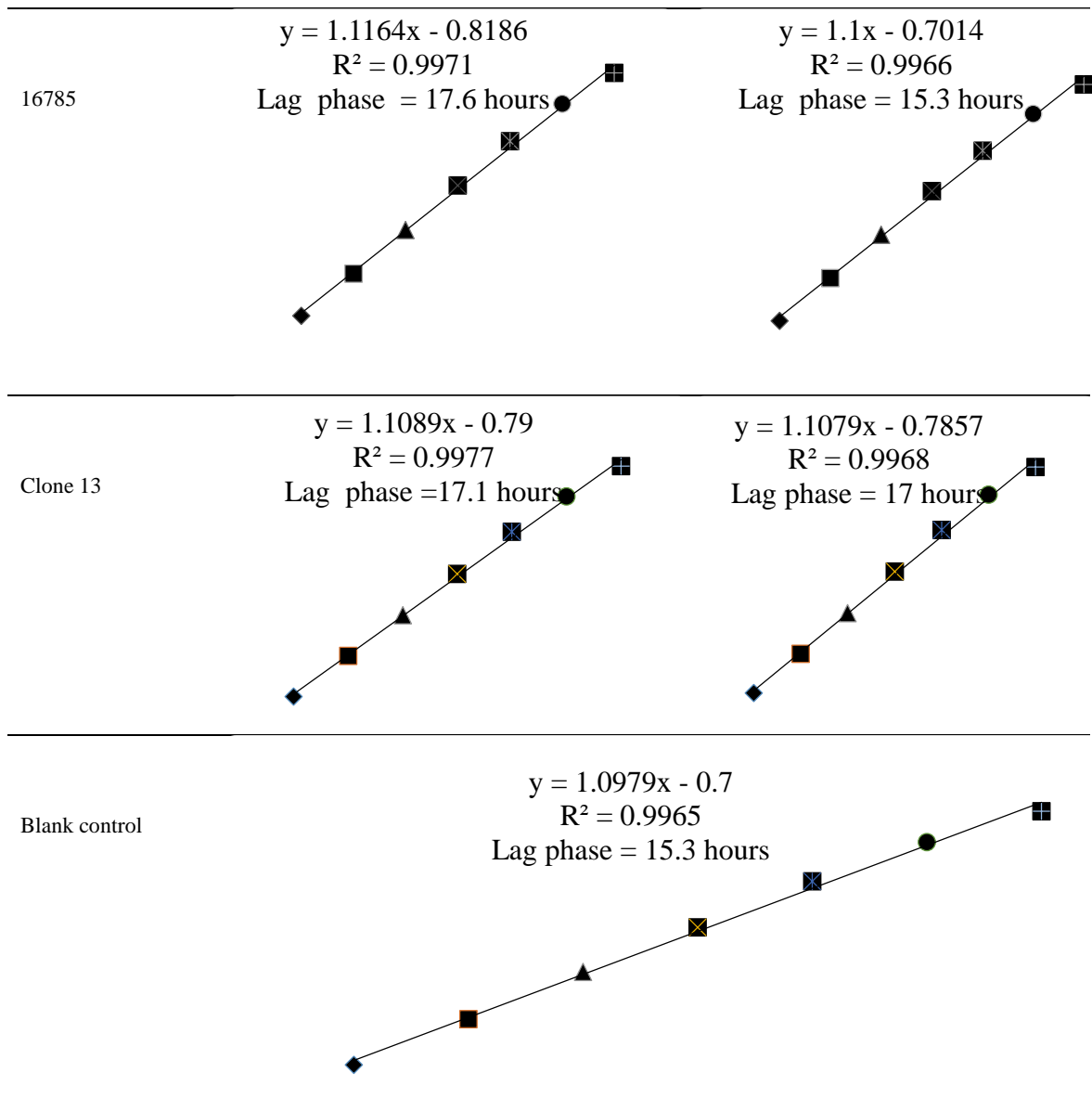Table 2. Analysis of variance results of the extracts treatments against the pathogen

Source of variation	df	Mean squares	F value	P value
Napier accessions	8	0.3290	2.96 **	0.0041
Extracts	1	0.6318	5.69 *	0.0183
Accessions × Extracts	7	0.0886	0.80 NS	0.5900

The notations (*$P \leq 0.05$, **$P \leq 0.01$) indicate the presence of significant effects, whereas the initials (NS) denote "Not Significant".

Table 3. The effect *in vitro* of napier accession's mixture of xylem-parenchymatous and leaf tissue extracts on the lag phase of *U. kamerunensis* on its growth on malt extract agar at 25°C using its colony diameter in centimeters against time.

Napier Accessions	Column A charts; Extracts from inoculated samples	Column B charts; Extracts from uninoculated control samples
16805	y = 1.1107x - 0.8571 R² = 0.9969 Lag phase =18.5 hours	y = 1.1125x - 0.7414 R² = 0.9965 Lag phase = 17 hours
Kakamega 1	y = 1.1157x - 0.8471 R² = 0.9969 Lag phase = 18.2 hours	y = 1.0868x - 0.77 R² = 0.9968 Lag phase = 17 hours

16902

y = 1.1143x - 0.8343
R² = 0.9971
Lag phase = 18 hours

y = 1.1164x - 0.7543
R² = 0.9967
Lag phase = 16.2 hours

16808

y = 1.0864x - 0.8143
R² = 0.9961
Lag phase = 18 hours

y = 1.0811x - 0.7557
R² = 0.9967
Lag phase = 16.8 hours

16811

y = 1.0825x - 0.7871
R² = 0.9949
Lag phase = 17.5 hours

y = 1.0932x - 0.7829
R² = 0.9975
Lag phase = 17.2 hours

16783

y = 1.1143x - 0.8071
R² = 0.9974
lag phase = 17.4 hours

y = 1.0764x - 0.7271
R² = 0.9958
Lag phase = 16.2 hours

16785	y = 1.1164x - 0.8186 R² = 0.9971 Lag phase = 17.6 hours	y = 1.1x - 0.7014 R² = 0.9966 Lag phase = 15.3 hours
Clone 13	y = 1.1089x - 0.79 R² = 0.9977 Lag phase =17.1 hours	y = 1.1079x - 0.7857 R² = 0.9968 Lag phase = 17 hours
Blank control	y = 1.0979x - 0.7 R² = 0.9965 Lag phase = 15.3 hours	

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