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Predicting Endophytes Contribution *In vivo* in Napier Grass Accessions' tolerance against *Ustilago kamerunensis* Using *In vitro* Strategies

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ABSTRACT

Two completely randomized laboratory assays were carried out at the plant pathology section of the National Agricultural Research Laboratories-Kabete in Nairobi Kenya. The aim of the studies was to determine the abundance of endophytes among an initially selected and presumed tolerant napier grass accessions to head smut pathogen and to decipher their possible synergistic or individualistic contribution to the accessions asymptomatic response to the disease challenge. The analyzed Shannon diversity indices results indicated that the abundance of fungal endophytes was unequal with some exceptions. Moreover, low inhibition percentages were obtained of the morphotypes in dual cultures *in vitro* with the pathogen, besides most of their interactions favouring the luxurious growth of the pathogen. Therefore, direct role of the endophytes to the tolerance of the accessions seems non-existent if not minimal. However, heightened hormonal secretions like auxins by the crop need to be investigated to determine whether the endophytes are enhancing its production to enable the plants tolerate the pathogen damage through compensatory growth strategies

Keywords: *Endophytes, Ustilago kamerunensis, Abundance, Inhibition, Dominance*.

INTRODUCTION

Mechanisms of resistance in plants against pathogens are primarily based on internal mechanisms which are genome based (Freeman and Beattie, 2008). However, a scenario exists where generally the fitness of a plant to survive a disease challenge is enhanced through secondary means; where a host plant's physiology and defense is restructured by some harmless microbes that survive within the plant's tissues popularly known as endophytes in what can be described as secondary strategy of tolerance (SST) (Herre, 2007; Rodriguez, 2009; Higgins, 2010). These microbes grow systemically in most plants where they secrete certain beneficial compounds to a plant's defense (Rodriguez, 2009). Hence, providing a pathway of pathogen's suppression by an infected plant in natural biological control mechanism.

These secretions can be inhibitory (Rachid and Mohamed, 2010), or can just enhance the host metabolism by triggering production of auxins that enhance the host's fitness in growth to cope with the disease establishment in poaceae family (Tanaka, 2012). Napier grass (*Pennisetum purpureum)* a poaceae is currently threatened by napier head smut disease caused by *Ustilago kamerunensis*. This disease causes significant biomass losses of up to 46% (Farrell, 2000). The infected plant's stems harden

and produce smutted premature flower, becoming thin and grassy. The subsequent regrowth is smaller and total dry matter of the affected crop reduces massively leading to reduced herbage yield (Farrell, 2002; Mwendia, 2007).

Therefore, to mitigate this disease challenge effectively through host plant resistance and biological control tactics there has been a need to identify if a possible secondary strategy of tolerance (SST) is involved by the accessions to the disease and possibility of head smut pathogen's antagonists existence for breeding and bio- prospecting purposes. Basing on this, the present study was conducted to try establish the likely role played by the endophytes isolated from an asymptomatic sample of napier grass crop to head smut bulked at KARI-Muguga South.

MATERIALS AND METHODS

A group of six purposively sampled ex-ILRI accessions presumed to be tolerant to napier head smut (table 1) which had been identified by Omayio . (2014), bulked at Kenya Agricultural Research Institute-Muguga South, located on (1º 13' 53.0" S) and (36º 38' 1.1" E) of Kiambu County in Kenya were used for this study. The 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 . (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 endophytes (Mwendia, 2006).

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

Napier accessions	Percentage dry matter/ Remark	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 . (2014)

Isolation of endophytic fungi from the napier accessions' seed stock

Five complete health plant representatives with all the above ground tissues for each of the purposively sampled accessions on experimentation were randomly collected from their bulking site at the research centre. The samples were then stored in clean plastic bags for delivery to the isolation laboratory at National Agricultural Research Laboratories-Kabete. A modified method was used as described by Arnold and Lutzoni (2007) and Higgins . (2010). At the isolation laboratory each accession's leaves and stems were washed randomly from the five plants with tap water thoroughly to remove surface dirt. The leaves were then cut into 2mm× 1mm pieces, which were then surface sterilized with 70% ethanol for 2 minutes, then 0.5% sodium hypochlorite for another 2 minutes with a final wash with 95% ethanol and deionized sterile water for 30 seconds respectively before drying them in between sterile paper towels. For the stems they were swabbed with 95% ethanol using sterilized cotton wool to avoid soaking of the sterilizing agent by the stem's soft vascular tissue that may eliminate the fungal endophytes. The stems were then cut into 2cm long cylinders between the nodes swabbed again before an eventual halving of the xylem-parenchymatous tissue cylinder lengthwise using a sterilized scalpel under a laminar air flow chamber. 1mm × 1mm dissected pieces from the quartered halves were then generated for the culturing in media. Complete sterility of the plant tissues was monitored by culturing the imprints of the sterilized leaf tissue on media and potential saprophytes growth monitored along with the main cultures for a potential action if it was necessitated by their contamination.

Culturing of the tissues, morphotypes identification and diversity estimation

The prepared accessions' leaves and vascular tissue pieces were then selected at random and plated in 2% malt extract agar which promotes growth of numerous endophytes (Higgins ., 2010). Twenty four plates in total per accession's tissue type (leaf tissue and stem tissue) were cultured in a completely randomized design. The plates were then sealed using a parafilm and incubated at 25ºC for 7 days in the dark. After the incubation the numbers of fungal endophyte morphotypes were then counted for each accession basing on their colony features viz. pigmentation on top, pigmentation on the reverse, texture and margin as per Sharma and Pandy (2010). The counts enabled the calculation of a Shannon weiner diversity indices (H') of the morphotypes for each of the accession on trial. The dominant six morphotypes across the accessions were then purposively sampled and their hyphae sub-cultured in six petri plates per morphotype for another 7 days at 25ºC in 2% MEA to enable measurement of their

absolute growth rate on media over the time of growth daily and to obtain pure cultures that were stored at 4^{o}C for the next tests described.

Inhibition tests between endophyte antagonists against Ustilago kamerunensis

The inhibition tests were conducted to study the interaction between *Ustilago kamerunensis* and the isolated six dominant endophytes for potential antagonistic ones. A modified dual culture method as described by Sharma and Trivedi (2010) was used. 2mm discs of the 7 day old sub-cultures of both the isolated dominant endophyte morphotypes and pathogen were then plated at opposite ends of a 9 cm petri plates 1cm from the petri edges for either culture. These set ups were sealed by a parafilm and replicated 10 times for each morphotype- pathogen dual culture in a completely randomized design. The plates were then incubated for 7 days at 25^{o}C along with control plates with only the pathogen. The radii of the radial colony growth were then measured and the percentage inhibition determined by the formula:

$$Percentage\ inhibition = \frac{r1 - r2}{r1} \times 100$$

Where: r1- is the radial growth of *Ustilago kamerunensis* control and r2 - is the radial growth of the pathogen in dual culture set up with the isolated fungal endophyte.

Index of dominance test between the pathogen and morphotypes in vitro

The competitiveness of the morphotypes against the pathogen basing on the nature of their interaction on dual culture set up as described above was then explored. The interaction type of each dual culture was examined macroscopically and index of dominance scores allocated as follows; 1 for mutual intermingling, 2 for mutual inhibition on contact, 3 for mutual inhibition at a distance, 4 dominance of one species on contact and 5 dominance at a distance.

Statistical analysis procedure

Shannon Weiner diversity indices were used to determine the abundance levels of the endophyte morphotypes in the accessions. Then a modified one tail-test of the Shannon diversity indices against each other as described by Zar (2010) was used through statistical analysis software version 9.1 to test the hypotheses of the presence or absence of equal abundance of endophytes across the presumed tolerant accessions. Testing for the presence or absence of significant differences on the radii of the pathogen in dual culture a two tail test at significance level of 5% was used.

RESULTS AND DISCUSSION

Abundance of isolated endophytes among the napier accessions determined

The respective endophyte morphotypes cultured out of the accessions' tissues exhibited the colony characteristics summarized on table 2. The colony forming units' tallies upon culture of the respective endophyte morphotypes from respective napier grass accessions were as demonstrated on table 3. The endophyte morphotypes' tallies revealed differences in the abundance levels of the endophytes among the respective accessions through the generated Shannon diversity indices (table 4). Kakamega 1 variety had the highest diversity index at 1.3440 followed by accession 16902 at 1.0918 from their leaf tissue cultures. Clone 13 variety and accession 16783 had the least Shannon diversity indices on their leaf tissues that stood at 0.6869 and 0.6853 respectively. Clone 13 a susceptible check exhibited a richer index than accession 16783 which had been selected as tolerant by Omayio . (2014). On the other hand, variations were observed on the stem tissues' indices, where accession 16902 had the highest index at 1.3252 followed by accession 16783 at 1.0618. In the stem tissues' analysis, 16808 had the least index at 0.3488 and Clone 13 was second last with an index of 0.6365. The presence of higher Shannon diversity indices on leaf tissue of some of the accessions compared to their stem tissues, could be attributed to the synchronous growth between them and the broadening of the leaves which the endophytes try to attain at very high rate leading to concentration of their mycelia on these leaf tissue regions. However, their mycelia can also be found in other regions of the plant like the stems a scenario that has been observed in other members of poaceae (grass) family (Tanaka ., 2012). Furthermore, the differences observed in the different napier grass accessions under test in their abundance levels could be attributed to the acquisition of the accessions from different regions of the world as reported by Lowe . (2003), which usually affects the incidence of endophytes making them highly variable within their populations similar to observations which have been made in native grasses of South America (Iannone, 2012).

Table 2. Colony characteristics of the isolated fungal endophytes and the pathogen U. kamerunensis

Fungal morphotypes	Colony characteristics on malt extract agar Surface colour	Reverse colour	Zonation	Texture	Growth rates (cm/day) in vitro at 25ºC
White 1 Endophyte Morphotype (W1EM)	White	White	None	Light cottony	0.6923 (R^2= 0.9980)
White 2 Endophyte Morphotype (W2EM)	White Margins Black Centre	Pale White	Wrinkled Reverse	Powdery	0.9155 (R^2= 0.9989)
White 3 Endophyte Morphotype (W3EM)	White	White	None	Floccose	Less dominant
Light Pink Endophyte Morphotype (LPEM)	Light Pink	Pink	Wrinkled Reverse	Fine Floccose	1.0411 (R^2= 0.9992)
Orange Endophyte Morphotype (OEM)	Orange	Orange	None	Fine Floccose	0.8917 (R^2= 0.9977)
Green Top Endophyte Morphotype (GTEM)	Green	Dark Green	None	Fine Floccose	0.5071 (R^2= 0.9742)
Light Grey Endophyte Morphotype (LGEM)	Light Grey	Pale Cream	None	Fine Floccose	0.4107 (R^2= 0.9918)
Green White Endophyte Morphotype (GWEM)	Green	White	None	Velvety	Less dominant
(Pathogen) Ustilago kamerunensis	White	Pale Cream	None	Floccose	1.0798 (R^2= 0.9943)

* Less dominant morphotypes were not among the six selected for evaluation in dual culture against Ustilago kamerunensis due to their low frequencies (table 3).

Table 3. The tally of specific endophyte morphotypes' colonies as identified on media

Napier grass accession	Leaf tissue culture Fungal endophyte morphotypes	Tally	Stem tissue culture Fungal endophyte morphotypes	Tally
	W1EM	20	LGEM	17
16902	W2EM	18	GTEM	22
	OEM	15	W1EM	13
			OEM	8
	OEM	10	W1EM	16
16808	LGEM	7	GWEM	2
	W1EM	14		
	LPEM	12	OEM	5
16811	W1EM	18	W2EM	19
	W2EM	4	LGEM	11
	W1EM	21	OEM	2
16805	W2EM	17	LPEM	6
			W3EM	4
	W1EM	22	OEM	8
16785	W2EM	14	W1EM	10
	LGEM	5	LGEM	4
	W1EM	18	W1EM	11
16783	W2EM	14	W2EM	17
			OEM	9
	W1EM	10	W1EM	4
Clone 13	LGEM	8	GTEM	2

A general one tail test of hypothesis on the accessions' leaf tissues and stem tissues endophyte abundance indices indicated a highly significant differences at (df = 7; t = 11.1375, p <. 0001) and (df = 7; t = 8.1204; p <. 0001) respectively. Further, a modified one tail-test of the Shannon diversity indices against each other as described by Zar (2010), indicated the presence of significant differences ($p < 0.05$) on endophyte abundance across the accessions indices (table 4). However, exceptions were noted with the following accession's leaf tissue indices pairing against each not exhibiting any differences (had equal abundance of the fungal endophytes) namely: Clone 13 versus 16783, 16785 versus 16811, 16805 versus 16783 and 16805 versus Clone 13; at (df = 41; t = 0.1074; P = 0.4575), (df = 43; t = 0.0163; P = 0.4935), (df = 63; t = 0.1901; P = 0.4249) and (df = 34; t = 0.0515; P = 0.4796) respectively. As observed on hypothesis testing on the presence of either equal or unequal abundance of endophytes among the accessions (table 4). Presumed tolerant accessions 16805 and 16808 had equal abundance of endophytes in the leaf tissues with susceptible variety Clone 13 which was converse of what was expected; which was the susceptible

(smutting) accessions to exhibit very low abundance from the presumed tolerant accessions if at all the endophytes exerted synergistic effect on inhibiting the pathogen in the accessions. Moreover, the morphotypes observed across the accessions were more or less the same (table 2).

Table 4. Shannon diversity indices of respective accessions in their respective tissues

Accession	Leaf Tissues' Shannon's diversity Indices	Rank	Stem Tissues' Shannon's diversity indices	Rank
Kakamega 1	1.3440 a	1	0.9810 e	5
16902	1.0918 b	2	1.3252 a	1
16808	1.0600 c	3	0.3488 h	8
16785	0.9576 d	4	1.0362 c	3
16811	0.9561 d	4	0.9734 f	6
16805	0.6876 e	5	1.0115 d	4
Clone 13	0.6869 e	5	0.6365 g	7
16783	0.6853 e	5	1.0618 b	2

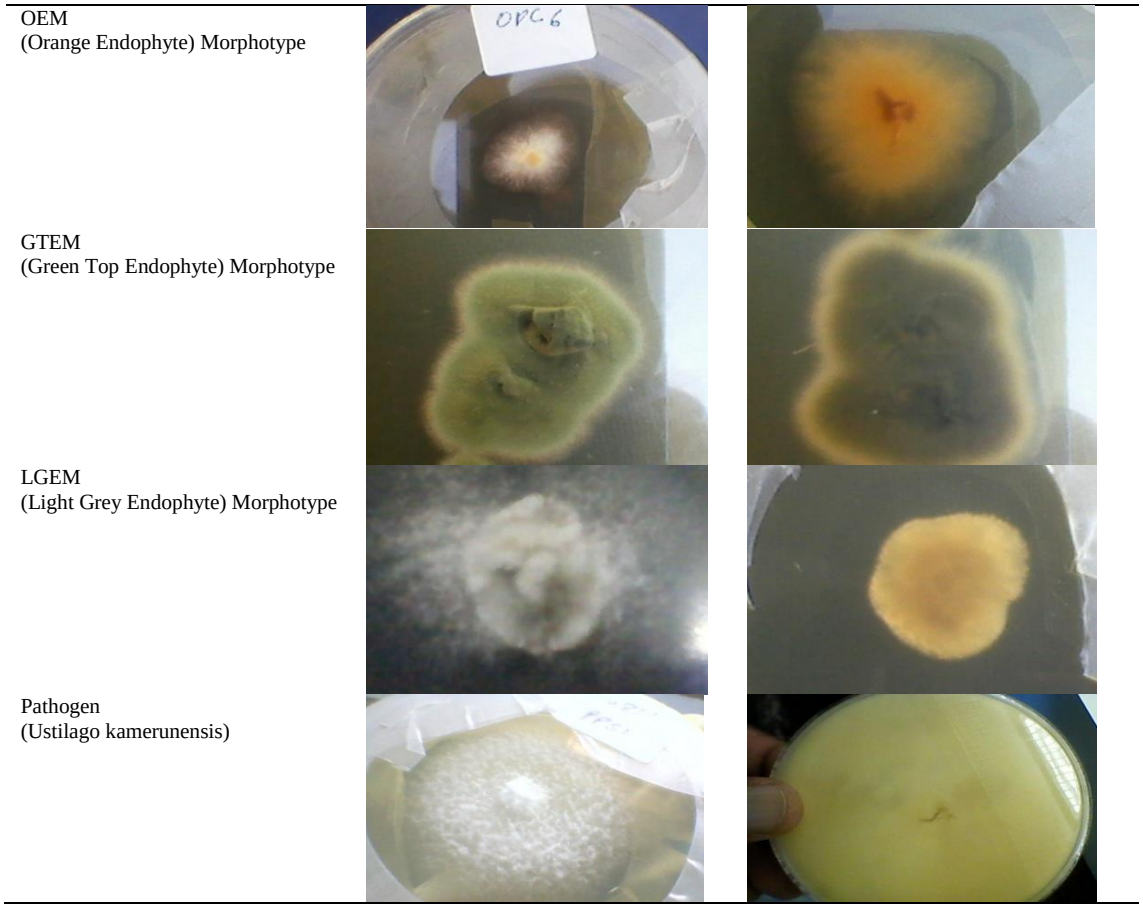
*Shannon diversity indices if followed by the same letter in the same column are not significantly different at $P \leq 0.05$ with a modified one tail-test as per Zar (2010)

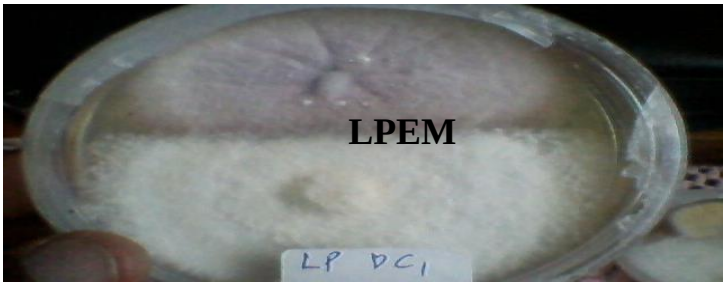
Selected dominant endophytes' inhibition tests against the U. kamerunensis

Dual culture experiments of the selected six dominant endophytes' (table 5) mean radii in dual culture treatments with the pathogen U. kamerunensis as shown on figure 1, demonstrated the presence of significant differences ($df = 5$; $t = 48.24$; $p < .0001$) on the pathogen's radii against the respective endophytes through a two tail test at significance level of 5% . However, using the formula indicated on table 6, the percentage inhibition levels determined revealed minimal levels of inhibition by the endophytes as summarized on the table 6. Endophyte morphotype (W2EM) had the highest percentage inhibitive level at 12.3457% followed by (LPEM) at 8.2305%. Morphotype (W1EM) had the least at 0.8230% with the overall mean inhibition levels of the endophytes being 5.4321%. The lack of an aggressive endophyte antagonist against the pathogen basing on the very low inhibition percentages observed, affirmed further the indications of a likely minimal role or none played by the endophytes on the resistance or tolerance of the napier accessions at individualistic level. Thus, reinforcing the earlier observations on table 4, where a higher abundance of endophytes was witnessed in Clone 13 (a susceptible variety) compared to 16783 an asymptomatic accession converse to what was expected.

Table 5. Pictures of the selected six dominant fungal endophytes and the Pathogen

Endophyte Morphotypes and the Pathogen	Picture of the Colony Top	Picture of the Colony Reverse
W1EM (White 1 Endophyte) Morphotype		
W2EM (White 2 Endophyte) Morphotype		
LPEM (Light Pink Endophyte) Morphotype		





Ustilago kamerunensis

Figure 1. Seven day old dual culture of endophyte morphotype (LPEM) against the pathogen U. kamerunensis

Table 6. Percentage inhibition levels of the pathogen by respective endophyte morphotypes

Pathogen's mean radius [r1 (cm)]	Pathogen's mean radii in dual culture with respective morphotypes [r2 (cm)]		Formula	Percentage Inhibition
2.43	W2EM	2.13	[(r1-r2)/r1] × 100%	12.3457%
	LPEM	2.23		8.2305%
	OEM	2.33		4.1152%
	LGEM	2.39		1.6461%
	W1EM	2.41		0.8230%

Index of dominance test between the endophytes versus the U. kamerunensis

The dominance test revealed a relatively balanced scenario of dominance as shown on figure 2 and table 7. There was no overwhelming dominance of the endophytes over the pathogen U. kamerunensis. A proportion of 17% of the isolated dominant endophytes dominated over the pathogen in a characteristic "engulfing" aggressive growth in vitro, whereas the pathogen dominated on 17% other isolated endophyte morphotypes (territorial antagonism). Further, U. kamerunensis intermingled freely

with 33% of some of the endophytes while inhibiting others on contact (fungistatic trophic antagonism). This lack of an outright aggressive endophyte antagonist against the pathogen Ustilago kamerunensis, based on the significantly low inhibition percentages that were observed in the dual culture experiments as indicated on table 6. In addition, the failure to observe the expected aggressive antagonistic types of interaction that is; mutual inhibition at a distance (mutual antagonism) and dominance at a distance (territorial antagonism) as illustrated in Andrea . (2005) in the index of dominance test. Such a phenomenon could be due to low or non-production of inhibitive factors by the endophytes against the pathogen, or the pathogen (U. kamerunensis) being resistant to the endophyte inhibitive factors a case example that has been observed in the evaluation of fungal endophytes for potential bicontrol agents against Rhizoctonia solani (Rachid and Mohamed, 2010). As result, this gave room for the pathogen to grow luxuriously in vitro to contact level with the involved endophytes in dual culture. Thus, ruling out the likely contribution of the endophytes to the tolerance in vivo.

This study concludes that there are differing abundance levels of the endophytes among the napier grass accessions. Though, their synergistic contribution in vivo to the resistance of the crop is minimal if not non-existent basing on the abundance levels of the endophytes isolated from susceptible variety Clone 13 in comparison to the isolations from the other selected tolerant accessions viz. 16783, 16785 and 16808. Moreover, the very low percentage inhibition levels and interaction types observed favouring the pathogen's luxurious growth in dual culture in vitro complements the conclusion and rules out individualistic involvement of the endophytes in inhibiting the pathogen. However, the contribution of the endophytes to the tolerance cannot not be ruled out in totality. This is because other host metabolism enhancement secretions by the endophytes have been reported like auxins that do not necessarily inhibit the pathogen but enhance the host's fitness in growth to cope with the disease establishment in poaceae (Tanaka, 2012). Hence, the need for comprehensive investigation into the likely roles of these morphotypes in the napier grass accessions.

Table 7. Pivotal table presentation of the various interaction types witnessed on trial

Morphotype	Index of Dominance Test Dominance of One Species on Contact (Index 4)	Mutual Inhibition on Contact (Index 2)	Mutual Intermingling (Index 1)	Grand Total
GTEM			10	10
LGEM			10	10
LPEM		10		10
OEM		10		10
W1EM	*10			10
W2EM	10			10
Ustilago kamerunensis			10	10
Grand Total	20	20	30	70

*The asterisk above denotes the endophyte morphotype (W1EM) that was dominated over in vitro by the pathogen U. kamerunensis

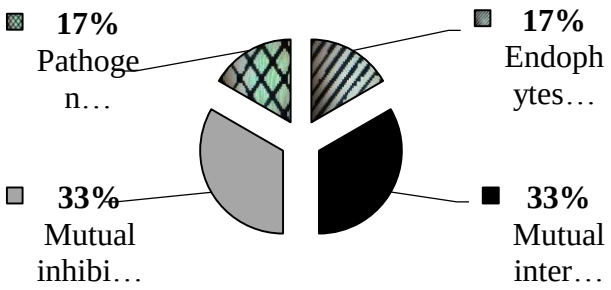


Figure 2. Percentage of predominant interaction types among isolated dominant endophyte morphotypes versus Ustilago kamerunensis

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