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The effects of calcium fertilizer on anthracnose and *Rhizopus* tuber rot of yam

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ABSTRACT

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Yam (*Dioscorea* spp) production is beset in the field and in storage by major fungal pathogens, namely *Colletotrichum gloeosporioides that* causes anthracnose in the field, and *Rhizopus stolonifer* that causes tuber rot in storage. Alternative control measures are imperative as conventional control of these pathogens is either ineffective or detrimental. This study investigated the effects of calcium fertilizer on the incidence and severity of anthracnose and *Rhizopus* rot of yam. Calcium nitrate fertilizer was applied to soils naturally infected with *C. gloeosporioides*, at the rates of 0, 3, and 4kg ha^{-1}. *D. rotundata* variety *Efuru* was planted. The treatments were arranged in a randomized complete block design with three replicates. Anthracnose disease rating relied on natural endemic infection in the field, while inoculation of harvested yam tubers was carried out using *R. stolonifer*. There was no significant effect of calcium amendment of the soil on anthracnose severity in *D. rotundata* or on the agronomic parameters. *D. rotundata* tubers treated with 3 and 4kg ha^{-1} calcium soil amendments had reduced infection by *R. stolonifer* in storage compared to the control treatments. The study concluded that calcium applied to the soil had reduced storage losses by *R. stolonifer* in *D. rotundata* but had no influence on anthracnose severity in the field. Therefore, 3kg ha^{-1} of calcium nitrate applied to the soil can be used to improve *D. rotundata* tuber resistance to *R. stolonifer*, and is recommended for high-quality tuber storage.

Keywords: calcium nitrate, soil amendment, anthracnose, *Rhizopus stolonifer*, *Dioscorea rotundata*

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INTRODUCTION

Yam (*Dioscorea* spp) is a tuber crop that is grown as a vital food and cash crop in West Africa (Mignouna et al 2014). It is considered to be the most nutritious of the tuber crops grown in the tropics (Wanasundera and Ravindran 1994). Cultivation of yams in Nigeria has been affected by abiotic and biotic influences, of which diseases are an important factor (Ayanwuyi et al 2011). Yam tubers are often attacked in storage by rot pathogens, which can cause up to 50% losses of fresh matter in about 6 months (Osunde 2008). *Rhizopus stolonifer* has been identified as one of the most important pathogens of stored yam tubers (Nahunnaro 2008, Dania et al 2012), and white yam (*Dioscorea rotundata* Poir.) has been shown to suffer the most severe losses caused by storage rot pathogens (Dania et al 2012).

Rhizopus stolonifer (Ehrenb.) is a fungus belonging to the Zygomycota (*Incertae sedis*) group and is commonly referred to as black mold. It causes soft rot in tuber tissues, where the tissues turn brown due to the spread of fungal mycelia through the tissues. The cell walls eventually collapse causing the tuber to become soft (Nahunnaro 2008).

Aside from fungal storage pathogens, field diseases also affect the production of yam. The most important yam disease in the field is anthracnose, caused by the fungal pathogen *Colletotrichum gloeosporioides*, which has been reported to attack almost all cultivated species worldwide (Emehute et al 1998) and can cause losses of up to 80% in Nigeria (Green 1994).

Various methods have been utilized in an attempt to control fungal pathogens. Yam tubers have been successfully treated with fungicides (Amusa and Ayinla 1997), but the environmental and health hazards posed by these toxic chemicals have discouraged the extensive use of this option. Other methods of storing fresh tubers, such as cold storage (Osunde 2008), are expensive and difficult for local farmers to use (Gyamfi 2002).

Fertilization with nutrients has been used to increase resistance or tolerance to diseases in host plants (Agrios 2005, Zambolim et al 2001). Calcium has been shown to have a positive influence on host plants against certain diseases. Calcium treatments have been used to control the effect of rot fungi on apples and strawberries (Cheour et al 1990, Biggs 1999). Root rot and *Fusarium* wilt have also been controlled by applying calcium to avocados and tomatoes (Duvenhage and Kotze 1991, Woltz et al 1992). Studies have also shown that increased calcium content in potato tubers hindered the severity of bacterial rot (Ozgen et al 2003), and calcium fertilization reduced the damage caused by storage fungal pathogens on improved yam lines (Otusanya et al 2016). Therefore, this study was carried out, using a local white yam variety in South-Western Nigeria, to investigate the effect of calcium fertilizer applied to the soil on *Rhizopus* tuber rot and anthracnose.

MATERIALS AND METHODS

Source of Planting Materials

Planting materials of white yam (*Dioscorea rotundata*) var. Efuru was sourced from the Osiele market, a local market in Abeokuta, Ogun State, Nigeria. Healthy-looking seed yams of about 0.5kg in weight were selected and carefully cleaned before planting.

Planting Site and Experimental Design

The field experiment was conducted at the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta (FUNAAB). Water yams (*Dioscorea alata*) had been planted in the plot the previous year. The field layout was arranged in a randomized complete block design (RCBD), with three replicates. Each replicate plot consisted of three treatments, and each treatment consisted of 10 mounds, making 30 mounds in each replicate block and 90 mounds in the experimental plot. Each mound was roughly a meter in diameter and about 80cm high. Each treatment plot was 5.8m by 2.2m, having an area of 12.76m^2, and the total size of each treatment plot across replicates was 38.28m^2. The treatments employed were: control (no application of calcium fertilizer), 3kg ha^{-1} calcium fertilizer, and 4kg ha^{-1} calcium fertilizer. While calcium nitrate fertilizer ($Ca(NO_3)_2$) was used for this study, measurement of the required amount of calcium was determined by calculation to identify the amounts of calcium nitrate fertilizer that would provide 3kg ha^{-1} and 4kg ha^{-1} of calcium to the soil. Planting was carried out at the onset of the rainy season (April) in 2016.

Soil Analysis

Soil samples were obtained from the experimental plot before the mounds were established. Fifteen (15) core samples were taken from each replicate in a zigzag pattern. Following the procedure carried out by Page et al (1982), the auger was pushed into the earth in a slanted position, to a depth of about 20cm, to ensure that both top soils and inner soils were collected. The extracted soil samples were each placed in separate, clean polythene bags and labeled, and after transport from the field, were allowed to air dry. They were then sieved with a 2mm sieve and bulked according to their replications. Determination of pH and analysis of the labeled (according to replications) soil samples were carried out according to standard soil analysis protocols by Page et al (1982).

Fertilizer Application

The fertilizer was applied in the morning (between 6:30am and 8:30am), at 3 months after planting. The required amount of calcium nitrate required to attain 3kg ha^{-1} and 4kg ha^{-1} calcium equates to 1.23g and 1.64g $Ca(NO_3)_2$per m^2 respectively.

Calcium nitrate fertilizer was weighed out into sterile polythene bags and transported to the experimental plot, where a blunt stick was used to dig a furrow at a radial distance of 20cm from the yam roots on each mound. The furrow was fertilized by sprinkling the fertilizer all around it. The furrow was then covered up with generous amounts of soil. No fertilizer was applied to the control plots.

Incidence and Severity of Foliar Disease and Tuber Rot Infections

The incidence of foliar diseases on the yam plants in this experiment and the severity of the disease were observed over a 4-month period. The experimental plot was established in an area known to be endemic with the *C. gloeosporioides* pathogen to allow for natural field infection of the yam plants. This pathogen uses appressoria to break through the cuticle of the cell wall and invades host cells

(Ryder and Talbot 2015). Infection incidence was measured by visually observing the number of infected plants and the total number of plants in the treatment plot.

$$\text{Incidence}(\%) = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \text{x}100$$

Severity of foliar disease was visually assessed by observing infected leaves, petioles and vines to see the extent to which the disease had affected the foliage. Such information was then rated using a 5-point scale: 1=≤2%, 2=3% -5%, 3=6% -10%, 4=11% - 25%, 5=≥50%;

In which

1 = no infection/highly resistant (0≤2%),

2 = 1.00–2.00mm necrotic spots or 3%-5% foliar infection/moderately resistant (tolerant)

3 = 2.01–3.00mm necrotic spots or 6%-10% foliar infection/moderately susceptible

4 = 3.01–4.00mm necrotic spots or 11%-25% foliar infection/susceptible

5 = above 50% infection or necrotic spots larger than 4.00mm/highly susceptible.

Severity scores were assessed according to the yam anthracnose disease assessment diagram developed by Otusanya et al (2016).

Rhizopus stolonifer isolates were extracted from infected tuber tissues obtained from a previous year's experiment in the Federal University of Agriculture, Abeokuta (FUNAAB). Pure cultures of the pathogen were grown on acidified potato dextrose agar (PDA) in sterilized glass petri-dishes at 28°C, and 7-day old isolates were used for inoculation.

Inoculation of Tubers

Milk harvest inoculation

Yam tubers were harvested 5 months after planting and inoculated with *R. stolonifer*. Tubers were inspected to ensure the absence of infection and injury. They were then cleaned carefully by dislodging clods of soil that had stuck to them and wiping their surfaces with a methylated spirit (95% v/v isopropyl alcohol as the active ingredient), after which they were left to stabilize for 1 week. Three tubers from each treatment were then selected. The tubers were then weighed using a field scale. The middle portion of each tuber was sterilized using cotton wool soaked in methylated spirit, and a flame sterilized 5mm cork borer was used to make a hole at the disinfected site. The core of the hole was removed with the aid of flame sterilized forceps. A 4mm disc of the *R. stolonifer* culture was placed into the hole using a flame sterilized 4mm cork borer. The extracted core of yam tissue was used to plug the hole and sealed with petroleum jelly. The inoculated tubers were incubated for 4 weeks at 28°C and weighed again. A sharp knife was used to cut through the incision site, and the infected tissue was pared away onto an aluminum foil sheet that had already been weighed. The weight of the infected tissue was then

measured. Percentage weight loss was calculated, following the method of Otusanya and Jeger (1994):

$$\text{Percentage (\%) weight loss} = \frac{\text{A-B}}{\text{A}} \text{x} 100$$

Where A = Weight of tuber at the onset of the experiment
B = Weight of tuber at the time of assessment

Percentage (%) infection was calculated using the formula:

$$\frac{\text{C}}{\text{A}} \text{x} 100$$

Where C = Corrected weight of infected or diseased tissue
A = Weight of tuber at the onset of the experiment

Corrected weight of infected tissue was formulated using:

$$\frac{100\text{X}}{100 - \text{Y}}$$

Where X = Weight of infected tissue
Y = % weight loss of tuber

Inoculation of tubers at final harvest

Tubers harvested at the final harvest of the experiment were also inoculated with *R. stolonifer*. The same procedure used during the milk harvest inoculation was carried out on the tubers harvested at 8 months after planting. However, these tubers were allowed to stabilize physiologically for 3 weeks before inoculation was carried out.

Data Collection

Each of the 90 yam stands was evaluated for the following pathological and agronomic parameters: incidence and severity of foliar diseases, vine length, number of shoots, tuber number at final harvest, and tuber weight at final harvest.

Incidence and Severity of Anthracnose

The incidence of anthracnose on yam plants was observed visually, identifying the percentage of plants infected against each treatment plot's total number of plants. Severity of anthracnose was also observed visually, making use of a disease assessment diagram to identify the extent of damage the disease caused on the foliar portion of the yam plants.

Severity of Tuber Rot

Diseased tissue from each inoculated yam was extracted and weighed against the overall weight of the yam to ascertain the weight loss and damage caused by the tuber rot. Weight losses due to physiological processes were accounted for by using the corrected weight formula.

RESULTS AND DISCUSSION

Nutrient Status of the Soil at the Experimental Site

The soil in the experimental site was slightly acidic with a pH of 6.34, sandy-loam, and had a calcium concentration of 33.08cmol kg^{-1} (Table 1). Available nitrogen, phosphorus, potassium and magnesium were 0.97%, 12.60ppm, 0.44cmol kg^{-1} and 2.46cmol kg^{-1} respectively.

The soils in the experimental plot lacked the required nutrients for the growth and production of yam. Soils sampled in the experimental sites, at the onset of the experiment, had 33.08cmol kg^{-1} (0.0033%) of calcium, while the nitrogen level was 0.97%. O'Sullivan (2010) observed that the critical range of calcium required for *D. rotundata* was 0.5-0.9%, while the critical range of nitrogen was probably 2.9-4.0% or lower. Thus, there was no natural source of calcium that could influence the results of this study, apart from that which was provided in this experiment.

Table 1. pH, texture, and nutrient status of the soil at the experimental site

Parameters	Experiment Plot
Texture	Sandy-loam
pH (in H_2O)	6.34
Calcium (cmol kg^{-1})	33.08
Nitrogen (%)	0.97
Phosphorus (ppm)	12.60
Potassium (cmol kg^{-1})	0.44
Magnesium (cmol kg^{-1})	2.46

Effect of Calcium on Incidence and Severity of Anthracnose on Efuru Yam

The disease severity scores recorded on *D. rotundata* var. Efuru over four months ranged from 1.00 to 3.00 (Table 2). The severity of the disease increased with time. At 4 months after planting (MAP), severity scores in all treatments were 1.00. At 5 MAP, scores ranged from 1.00 to 1.33, with the 4kg ha^{-1} treatment having the lowest score of 1.00. At 6 MAP, scores ranged from 1.67 to 2.33, with the 3kg ha^{-1} treatment having the lowest score of 1.67 and the 4kg ha^{-1} treatment with the highest score of 2.33. At 7 MAP, scores ranged from 2.67 to 3.00, with the 3kg ha^{-1} treatment having a score of 2.67, while the control and 4kg ha^{-1} treatments had a score of 3.00. There were no significant (p>0.05) differences among treatments.

At 4 MAP, anthracnose incidence ranged from 10.00% in the 3kg ha^{-1} treatment to 16.67% in the control treatment. At 5 MAP, the incidence range increased, from 36.67% in the 3kg ha^{-1} treatment to 46.67% in the control treatment. At 6 MAP, the incidence ranged from 66.67% in the 3kg ha^{-1} treatment to 86.67% in the control treatment. All yam plants on the field were infected with anthracnose as at 7 MAP (incidence of the disease was 100% in all treatments).

Above-ground symptoms observed on affected yam plants included leaf spots of varying sizes, some of the larger ones having a “bulls-eye” design (necrotic spot with concentric circles). Most of the leaf spots had chlorotic “haloes” surrounding necrotic lesions. Blights, where irregular portions of the leaf turned necrotic, were also observed. There were also blotches ranging from whitish to brown, which were

brown on the underside of the leaf. Some leaves also showed various discolorations, most of which turned brown over time. The veins on the undersides of some leaves turned black, and such leaves tended to look somewhat chlorotic. The vines of some yams turned black, and small cankers appeared on some of them. Calcium is implicated in the formation of calcium pectate, which gives the cell wall its rigidity (Burstrom 1968). However, soil amendment with calcium nitrate did not reduce the incidence or severity of anthracnose in any of the treatments. This is likely due to the fact that calcium is an immobile nutrient. Since the calcium fertilizer was applied to the soil at the onset of tuber formation (3 MAP), calcium taken up by the yams may have been directed primarily to the development of the tubers. This would disallow much calcium from being translocated to the aerial portions of the yams. Since the soils used in this experiment were deficient in calcium, it is speculated that the calcium available to the plants was not enough to be distributed to all parts of the plants. This would result in tubers having a higher amount of calcium, while the foliar regions would have lower amounts. The amount of calcium that is actually translocated to the vines of yams during tuber formation could be measured in future researches to give a clearer understanding of how much calcium might be actually required by yam plants to ensure its availability in the vines.

Table 2. Effect of calcium fertilizer on incidence (%) and severity of anthracnose on *Dioscorea rotundata* var. Efuru

Treatments (kg ha^{-1}) Ca	4 MAP		5 MAP		6 MAP		7 MAP	
	I (%)	S	I (%)	S	I (%)	S	I (%)	S
0	16.67	1.00^{a}	46.67	1.33^{a}	86.67	2.00^{a}	100	3.00^{a}
3	10.00	1.00^{a}	36.67	1.33^{a}	66.67	1.67^{a}	100	2.67^{a}
4	13.33	1.00^{a}	43.33	1.00^{a}	76.67	2.33^{a}	100	3.00^{a}
Coefficient of variation	23.02	0	9.85	12.75	10.65	13.47	0	5.38

Means with the same letter among columns are not significantly different
I = Incidence; S = Severity
MAP = Months after Planting

Percentage Rhizopus Rot Infection and Yield Loss of Efuru Yam Tubers as Affected by Calcium Fertilization

The calcium treatments showed no effect on the percentage of *Rhizopus* rot infection of yam tubers harvested at 6 MAP, as well as no effect on the weight loss of tubers. At 8 MAP, however, tubers from plants treated with calcium nitrate had a significantly lower percentage of infection (0.57% and 0.61% for 3kg ha^{-1} and 4kg ha^{-1} treatments respectively vs 48.58% for the control) and a lower percentage weight loss (2.84% and 1.16% for 3kg ha^{-1} and 4kg ha^{-1} respectively vs 3.18% for the control; Table 3). However, weight loss values were not significantly different from each other. Tubers from plants treated with 3kg ha^{-1} also had a significantly lower percentage infection than those treated with 4kg ha^{-1} calcium nitrate.

Rhizopus stolonifer is a common pathogen causing rot in various plant products, and unlike phytopathogenic fungi that utilize appressoria to infect plant cells, *R. stolonifer* uses esterase enzymes, which break down the cell walls and allow the haustoria of the pathogen to access the cell (Baggio et al 2016). Calcium is implicated in the improving of plant resistance to both bacterial and fungal

pathogens. It was reported to be important for maintaining cell wall integrity in hydroponic grown carrot roots (Cho et al 2000). Also, Gunter et al (2000) reported that an increase in calcium content in sweet potato tubers reduced soft rot severity. In this study, a similar trend was observed in *D. rotundata* tubers treated with 3kg ha^{-1} and 4kg ha^{-1} calcium applied to the soil. The tubers showed reduced *R. stolonifer* rot infection compared with the untreated control treatment. A similar result was observed where calcium fertilization reduced infection and weight loss caused by *Aspergillus niger* and *Botryodiplodia theobromae* in improved varieties of yam (Otusanya et al 2016).

Young tubers harvested from the experimental plots, sampled for infection and weight loss at 6 months after planting (milk harvest), seemed to have a slower progression of *Rhizopus* rot infection than the more mature tubers from the plants that had turned senescent at 8 months after planting. *Dioscorea rotundata* tubers in the control experiment, which were highly susceptible to the rot pathogen at final harvest, had low percentage infections and weight losses observed during milk harvest sampling. This is likely to be due to a higher amount of phenolic substances present in young tubers. This line of thought is corroborated by findings that showed that the level of phenolic compounds in *D. rotundata* is higher in developing tubers than at tuber maturity or vine senescence (Hamadina and Craufurd 2015). Therefore, young tubers are likely to have a higher shelf life than mature tubers, and the implications of this knowledge can be further explored.

Table 3. Percentage infection and weight loss in *Rhizopus stolonifer-infected D. rotundata* var. Efuru tubers as affected by calcium nitrate fertilization

Treatments (kg ha^{-1}) Ca	6 MAP		8 MAP	
	% Infection	% Weight Loss	% Infection	% Weight Loss
0	1.09^{a}	15.98^{a}	48.58^{a}	3.18^{a}
3	1.12^{a}	16.50^{a}	0.57^{c}	2.84^{a}
4	1.90^{a}	18.16^{a}	0.61^{b}	1.16^{a}
Coefficient of variation	26.69	5.51	135.03	36.95

Means with the same letter among columns are not significantly different
MAP = Months after Planting
Transformed values are in parentheses

Effect of Calcium Nitrate Fertilizer on Agronomic and Yield Parameters

Vine lengths observed in the experimental plot ranged from 266.28cm to 309.58cm at 1 month after fertilization (4 months after planting), and 318.27cm to 373.33cm at 3 months after fertilization (6 months after planting). Plants treated with 3kg ha^{-1} calcium had the shortest mean vine lengths, while those treated with 4kg ha^{-1} had the longest mean vine lengths. However, there was no significant effect of the different calcium nitrate treatments on the vine lengths at either 1 month or 3 months after fertilization. The number of shoots ranged from an average of 2.27 to 2.77 at 1 month after fertilization and 2.57 to 2.93 at 3 months after fertilization (Table 4). Although in this case, the lowest mean number of shoots was observed in the control treatments and the highest mean number of shoots was observed in plants treated with 4kg ha^{-1}, the calcium fertilizer levels applied had no significant effect on the number of shoots produced per plant.

The number of tubers per plant, as well as the tuber weights per plant harvested from the experimental plot, was not noticeably affected by the treatments with calcium fertilizer used in this study (Table 4). The number of tubers ranged from an average of 1.84 to 2.87, with plants treated with 3kg ha^{-1} yielding the least mean number of tubers, while the highest mean number of tubers was recorded in plants treated with 4kg ha^{-1}. A similar trend was observed in the lowest and the highest mean weights of yam tubers harvested per plant. The weights of harvested tubers ranged from 3.01kg to 3.57kg per plant.

Calcium is an essential nutrient that is involved in many biochemical processes in plants involving bud formation, secretion, and hormone-regulated growth and development (Hepler and Wayne 1985). However, the results observed in this study showed that the calcium nitrate applied to the soil had no significant effect on the vine lengths or number of shoots of the yam plants. This is a result of a deficiency of nitrogen in the soil, as the field had been planted with yams in the previous year. Yams are heavy feeders and have been known to require very fertile soils to grow well (O'Sullivan 2010). Therefore, the calcium nitrate fertilizer applied was unable to meet the nitrogen requirement of yams that would significantly improve their agronomic or yield parameters.

The yield obtained from the *D. rotundata* plot in which calcium nitrate fertilizer was applied to the soil was 87.35kg from the 3kg ha^{-1} treatment and 107kg from the 4kg ha^{-1} treatment, making a total of 194.35kg. Extrapolating that amount gives an estimated yield from the fertilized plots of 32.39t ha^{-1} of calcium fertilized white yam tubers. According to the study by Diby et al (2009), the potential yield from 1 hectare for white guinea yam is 27t ha^{-1}. This implies that there was actually a positive effect of calcium fertilizer on the overall yield when compared with that study (Diby et al 2009), as the results of this study showed a 19.96% higher yield. The unfertilized plot (control treatment) yielded 94.4kg. Extrapolating this to estimate the yield of yam generated from the unfertilized plot gives 31.47t ha^{-1}. This is still higher than that of the yield reported by Diby et al (2009); however, in this experiment the yield obtained from the fertilized plot is 2.92% higher than that of the unfertilized plot.

Table 4: Agronomic and yield parameters of *Dioscorea rotundata* var. Efuru as affected by calcium fertilizer

Treatments (kg ha^{-1}) Ca	1 MAF		3 MAF		Harvest	
	Number of Shoots (per plant)	Vine Length (cm per plant)	Number of Shoots (per plant)	Vine Length (cm per plant)	Tuber Number (per plant)	Tuber Weight (kg per plant)
0	2.27[a]	277.80[a]	2.57[a]	340.30[a]	2.47[a]	3.15[a]
3	2.31[a]	266.28[a]	2.63[a]	318.27[a]	1.84[a]	3.01[a]
4	2.77[a]	309.58[a]	2.93[a]	373.33[a]	2.87[a]	3.57[a]
Coefficient of variation	9.27	6.44	5.81	6.58	17.74	7.34

Means with the same letter among columns are not significantly different

CONCLUSION

The findings in this study concluded that calcium nitrate applied to the soil had no effect on anthracnose in the field, nor did it cause any changes in the number of shoots and vine lengths in *Dioscorea rotundata*. There was also no effect of calcium fertilizer at 3kg ha^{-1} or 4kg ha^{-1} on the percentage infection by *Rhizopus stolonifer* and weight loss in any of the young tubers harvested at 6 MAP, although the percentage of infection and weight loss were generally low. However, the more mature tubers showed a higher level of damage caused by *Rhizopus stolonifer*, and 3kg ha^{-1} and 4kg ha^{-1} of calcium nitrate applied to the soil was found to reduce storage losses caused by this pathogen in *D. rotundata*. It is therefore recommended that 3kg ha^{-1} of calcium nitrate (due to being more cost effective than 4kg ha^{-1}) can be applied to the soil to improve the shelf life of fresh white yam tubers by reducing infections by *Rhizopus stolonifer*.

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