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Influence of Nitrogenous and Phosphatic Fertilizers Types and Rates on the Yield and Chemical Profile of *Sutherlandia frutescens* and *Leonotis leonurus*

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Abstract

Sutherlandia frutescens and *Leonotis leonurus* are two important medicinal plants used traditionally to treat diabetes and inflammation harvested mainly from natural resources. The shift from subsistence to commercial trade necessitates research into cultivation, and hence information is needed for commercial production of these species. The effects of nitrogen (N) and phosphate (P) fertilizers in different forms and also at different levels on the growth and yield as well as the chemical composition were investigated. This study reported fertilizer applications' dramatic response on growth and development. The results provide a foundation for more in depth cultivation research and guidelines for soil amendment in increasing yield under field production. For *L. leonurus* ureum applied at 360 kg N ha^{-1} resulted in the highest yield. The highest yield for *S. frutescens* was obtained with ammonium sulphate at 360 kg N ha^{-1}. No significant improvement in yield was obtained with P amendment for *L. leonurus* while *S. frutescens* could not survive the P trial conditions and most of the plants died. Thin layer chromatography (TLC) provided an overview of the effect of soil amendment on the chemical profile of the plants with various differences observed on the TLC plates by comparing the sample spots. Incomplete information on the active principles of both species poses various challenges in determining the effect of soil amendment on quality. These results warrant further studies into the effect of the changes in the chemical profile on the medicinal activity of the plants and specifically on target compounds. © 2017 Friends Science Publishers

Keywords: *Sutherlandia frutescens*; *Leonotis leonurus*; Nitrogen; Phosphorus; TLC

Introduction

In spite of increasing urbanization, a large proportion of the African population has retained their reliance on traditional medicine for primary health care. This leads to medicinal plant harvesting changing from plant harvesters with the primary aim of making a profit, to a specialist activity (Wiersum *et al.*, 2006). Plants with traditional use are of interest to many scientists as it reveals their potential to be exploited to treat various diseases (van Wyk and Albrecht, 2008). Two important medicinal plants used traditionally to treat diabetes, inflammation and with an effect on the central nervous system have been investigated in this study (Albrecht *et al.*, 2012; Nsuala *et al*., 2015).

Sutherlandia frutescens belongs to the family Fabaceae commonly known as the legume, pea or bean family. It is commonly known as the cancer bush because of the reported use by Khoi-San and Cape Dutch people against internal cancers since 1895 (Diederichs, 2006), but also known as kankerbos, gansies, grootgansies, keurtjie, rooikeurtjie (Afrikaans), Blasenstrauch, Krebsbusch, Sutherlandia (German); musa-pelo, musa-pelo-oanoka, motlepelo, (Sesotho), phetola (Setswana), insiswa and unwele (isiZulu, isiXhosa) (van Wyk and Albrecht, 2008). The plant is recognised by the large red flowers, around 3 cm long, followed shortly by bladder-like fruits (van Wyk *et al.*, 2000), but mainly the leaves are used medicinally although all above ground parts are often included to treat stomach ailments, backache, diabetes, stress, fever, wounds (Drewes *et al*., 2006) and a general tonic (Crouch and Symmonds, 2006). The first small-scale cultivation and commercialisation of *Sutherlandia* started in the Cape Province more than 20 years ago (Diederichs, 2006), but the production is not meeting the demand (Albrecht *et al.*, 2012). Some compounds such as L-canavanine, GABA, and D-pinitol have been identified in the plant and linked to some aspects of its medicinal use, but the geographical effects on the chemical profile prevented the development of any guidelines or

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chemcial profiling of the plant (Acharya *et al.,* 2014).

Leonotis leonurus or Wild dagga belongs to the family Lamiaceae and has a distribution over large parts of South Africa (van Wyk and Gericke, 2001). It is a robust shrub which grows up to 2-3 m tall and 1.5 m wide. The stems are velvety and woody at the base with long, narrow and rough leaves with serrated edges. It is known by different names in different cultures that include Lebake (Sotho), Umfincafincane (Xhosa) and Wilde dagga (Afrikaans). This plant has been the subject of numerous studies to validate the use of the plant in traditional medicine. The anticonvulsant (Bienvenu *et al.,* 2002), antifungal (Motsei *et al.,* 2003), antiplasmodial (Clarkson *et al.,* 2004) and antibacterial activity (Kelmanson *et al.,* 2000) are some of its attributes that have been investigated. The leaves are smoked for the relief of epilepsy and an infusion or decoction of the leaves and stems used internally for coughs, colds, influenza, bronchitis, high blood pressure and headaches. Externally, decoctions have been applied to treat boils, eczema, skin disease, itching and muscular cramps (van Wyk and Gericke, 2001). It has also been used as an emetic for snakebite and infusions of the leaves for dysentery and colds (Kelmanson *et al.,* 2000). Limited information is available on the chemical constituents and pharmacological activity of compounds of *L. leonurus* although some compounds such as sterols, diterpenes, triterpenoids, tannins, flavonoids, alkaloids, quinines and saponins have been identified (Maphosa and Masika, 2008).

Plants such as *L. leonurus* and *S. frutescens* harvested in large numbers from the wild are at risk of losing genetic diversity due to habitat destruction. The affordability, non-accessibility and increased rates of unemployment are additional factors which have resulted in the commercial exploitation of economically valuable plants by gatherers as a source of income (Cunningham, 1993). Domestic cultivation is a viable alternative and offers the opportunity to overcome the uncontrolled harvesting and exploitation of medicinal plants (Canter *et al.,* 2005). New strategies for developing these medicinal plants as commercial crops is needed and other possibilities such as incorporating the crops as an alternative for larger, commercial farms and for the small-scale farmer (Wiersum *et al.,* 2006). Cultivation data however needs to be linked with pharmacological assessments of the crop during the development of protocols. Levels of secondary metabolites in plant tissues have been reported to vary with environment, geographical region and nutrient availability (van Wyk and Albrecht, 2008; Colling *et al.,* 2010).

Application of N and P has resulted in varying and contradicting results in previous studies. Positive effects were observed in the growth and yield of medicinal species such as *Artemisia annua*, *Pelargonium graveolens* and *Cymbopogon martini* (Rao, 2001; Ram, 2003; Özgüven *et al.,* 2008). Application of N at 93.75 kg ha^{-1} gave the highest values of plant height, number of laterals, fresh and dry weight of shoot, dry matter production, fresh herb yield and essential oil yield of Davana (*Artemisia pallens*). In fennel, only ammonium sulphate was able to produce comparable swollen base yield compared to the control, while a significant reduction was obtained with ammonium nitrate and urea (Atta-Aly, 2001). *Cymbopogon martini* showed an increase in biomass by 42.6% with 40 kg N ha^{-1} and 57.6% with 80 kg N ha^{-1}. The essential oil yield also increased by 44.4% with 40 kg N ha^{-1} and 60.3% at 80 kg N ha^{-1}. Nitrogen fertilization had a significant increase in the oil content without any difference observed on the nutrient content of black cumin (*Nigella sativa* L.) seeds (Ram, 2003) stating the diverse and varied response of plants to fertilizer, also affected by the direct environment. In *Calendula officinalis* it has been found that increase in P did not increase the flower yield, but increased leaf biomass (Stewart, 2003).

This study is the first report on the effects of different fertilizer and their levels on the growth and development of *S. frutescens* and *L. leonurus* in the field. In this study, the effects of different N and P fertilizers on the growth, yield and chemical composition of two medicinal plant species have been studied. As there are no information currently available on the fertilizer needs of these plants, this study provide a sound foundation for further research on the effect of fertilizer on medicinal active compounds and how it affects the various medicinal properties of the plants.

Materials and Methods

Plant Material

Stem cuttings for the N and P trials were produced in April, from plants grown at the Agricultural Research Council (ARC)-Roodeplaat, Vegetable and Ornamental Plants (VOP), Pretoria, South Africa (25°59'S; 28°35'E and 1 200 m.a.s.l.). The stem cuttings were grown in a plastic tunnel irrigated twice a day for 15 min, without any temperature regulation. The cuttings that rooted by September were used for establishing the field trials in October of the specific year. Since the material is not commercially available, production of stem cuttings ensured plant material was of the same genetic origin and age.

N Trial Layout and Treatments

The trial was planted as a complete randomized block design with 15 treatments and three replicates. Each plot consisted of 28 plants with 10 data plants at a spacing of 0.7 m between rows and 0.5 m within rows, resulting in a plot size of 6.54 m^2. Limestone ammonium nitrate (LAN), ureum and ammonium sulphate (28%, 46% and 21% N respectively) were used as the three fertilizers applied at 0, 180, 240, 300 and 360 kg N ha^{-1}. Treatments 1–5 denotes LAN at 0–360 kg N ha^{-1}, 6–10 denotes ureum at 0–360 kg N ha^{-1} and 11–15 denotes ammonium sulphate at 0–360 kg N ha^{-1}.

Phosphorus Trial Layout and Treatments

The same trial layout was used as for the N trial with 15 treatments and three replicates. Each plot consisted of 28 plants with 10 data plants at a spacing of 0.7 m between rows and 0.5 m within rows, resulting in a plot size of 6.54 m^2. Superphosphate, ammonium supers and mono ammonium phosphate (MAP) were applied at 0, 30, 70, 100 and 120 kg P ha^{-1}. Treatments 1–5 denotes superphosphate at 0–120 kg P ha^{-1}, 6-10 denotes ammonium supers at 0–120 kg P ha^{-1} and 11–15 denotes MAP at 0–120 kg P ha^{-1}.

Soil Analysis and Fertilizer Application

The trial was established in October on a deep sandy soil classified as Fernleaf (Soil Classification Working Group, 1991). A soil analysis was performed on the soil before planting, and used for application of phosphorus and potassium (Table 1) for the N fertilizer trial. As the soil is non-fertile deep sandy, low nutrient levels were found with undetectable levels of N. The soil was prepared a month before planting by using a plough, disc and Vibroflex. The fertilizer was applied before planting and incorporated into the soil with a rotovator. Brassica fertilizer recommendation was used to apply MAP at 70 kg ha^{-1} and potassium sulphate at 120 kg ha^{-1}. Fifty percent (50%) of the nitrogen was applied directly after planting, 25% applied after four weeks and 25% in February of the following year.

For the P trial, the experimental field next to the area used for the N trial was utilized, which provided the same soil analysis as provided in Table 1. The same trial layout, number of plants and plot size were maintained. Brassica was again used as the norm and potassium sulphate was applied at 120 kg K ha^{-1} and ureum at 180 kg N ha^{-1}. Nitrogen was applied directly after planting, 25% applied after four weeks and 25% in February of the following year.

Harvesting

Harvesting was planned for February and April, but due to the slow response of *S. frutescens* to the N and P fertilizer amendment and the poor response of *L. leonurus* to P, these trials were only harvested once in April. The *L. leonurus* N trial was harvested twice as the plant growth was very fast and the data of the harvests were combined to obtain a total yield for the trial. The aerial parts of the plants were removed 20 cm from the ground and all the plant material weighed to determine the fresh yield. For each treatment, a number of 10 plants were used as data plants and an average fresh yield was calculated. An average was also calculated for the three replicates before the data was statistically analyzed.

Data Analysis

The data was analyzed by the ARC-Agrimetrics Biometry Unit by using the statistical program Genstat (2000). If the probability of the main effects was significant at 5%, the treatment averages were separated by using Fisher's protected t-test of the least significant differences (LSD).

Extract Preparation

Plant material was air dried at room temperature. The dried material was weighed out and the samples prepared for extraction with two different solvents. An ethyl acetate and methanol extract was prepared for chromatographic analysis to extract both polar and non-polar compounds. The extracts were prepared by grinding 20 g plant material in a grinder (IKA-Werke M20, Germany) and adding 150 mL of the respective solvent at room temperature. It was stirred with a magnetic stirrer bar for 15 min, filtered and dissolved in another 150 mL solvent. The filtered extracts were combined to yield 300 mL of extract concentrated under reduced pressure. The dried extract was then used for chromatographic analysis.

Chromatographic Analysis

Aluminium Thin Layer Chromatography plates (20 x 20 cm) covered with silica gel (Merck 60 F_{254}, 0.2 mm thickness) was used. A 10 mg/mL solution was prepared for each extract. Using a TLC syringe 5 µL of each extract was spotted on aluminium TLC plates developed with a mobile phase of 95 chloroform: 5 methanol. A vanillin solution was used as a colour reagent, prepared by adding 7.5 g vanillin (Sigma) to 250 mL methanol and 5 mL H_2SO_4.

Results

All the N treatments showed significant increases in the yield of the plants when compared to the controls. *Leonotis leonurus* is a much easier and better growth obtaining yields of 42.17 t ha^{-1} (Table 2), whereas *S. frutescens* could only reach 12.85 t ha^{-1} (Fig. 1), even at the highest levels of fertilizer application. All treatments of N, however, significantly increased the yield of the fresh mass when compared to the controls. For *L. leonurus* a fertilizer level of 300 kg N ha^{-1} applied as ureum was sufficient to increase the yield significantly compared to the lower fertilizer levels and the control with a yield of around 13 t ha^{-1} (Table 2). Ureum produced the highest yield at the highest fertilizer level of 360 kg N ha^{-1}, but it was not significantly better than the lower applications. The yield was much lower for *S. frutescens* than for *L. leonurus* with the highest yield of 13.5 t ha^{-1} for the 360 kg N ha^{-1} ammonium sulphate treatment compared to the yield of the control of 0.77 t ha^{-1}, which did not reach saturation levels yet and might still increase as an almost linear increase is observed to the highest application level.

Leonotis leonurus reached the highest yield of 24.56 t ha^{-1} with addition of 100 kg P ha^{-1} compared to the

Table 1: Soil analysis used for recommendation of nitrogen, phosphorus and potassium

Method	P-Bray-1		Amm. Acetate								Water
	P	K	K	Ca	Ca	Mg	Mg	Na	Na	R	pH
	mg/kg	mg/kg	me/100 g	mg/kg	me/100 g	mg/kg	me/100 g	mg/kg	me/100 g	ohm	
	22.70	86.00	0.22	432.00	2.16	108.00	0.89	3.70	0.02	2210.00	6.59

Table 2: Influence of N fertilizer types on the first harvest, second harvest and the total harvest calculated in t ha-1 for *L. leonurus* (LSD 0.05)

	Yield in t ha^{-1}		
Treatments	1st harvest	2nd harvest	Total
LAN 0	8.57 ^{c}	4.97 ^{efg}	13.54 ^{de}
LAN 180	17.43 ^{abc}	8.09 ^{bcde}	25.51 ^{cd}
LAN 240	22.80 ^{ab}	9.71 ^{abc}	32.51 ^{abc}
LAN 300	24.63 ^{a}	10.66 ^{ab}	35.29 ^{abc}
LAN 360	26.71 ^{a}	9.91 ^{abc}	36.63 ^{abc}
Ureum 0	9.31 ^{bc}	4.17 ^{fg}	13.49 ^{de}
Ureum 180	19.34 ^{abc}	8.49 ^{bcd}	27.83 ^{bc}
Ureum 240	25.17 ^{a}	7.17 ^{cdef}	32.34 ^{abc}
Ureum 300	29.14 ^{a}	11.14 ^{ab}	40.29 ^{ab}
Ureum 360	29.63 ^{a}	12.54 ^{a}	42.17 ^{a}
Ammonium sulphate 0	7.09 ^{c}	3.51 ^{g}	10.60 ^{e}
Ammonium sulphate 180	28.40 ^{a}	5.91 ^{defg}	34.31 ^{abc}
Ammonium sulphate 240	24.11 ^{a}	7.20 ^{cdef}	31.31 ^{abc}
Ammonium sulphate 300	23.06 ^{a}	10.03 ^{abc}	33.09 ^{abc}
Ammonium sulphate 360	24.63 ^{a}	7.23 ^{cdef}	31.86 ^{abc}
LSD $_{0.05}$	13.66	3.27	13.70

*LSD: least significant difference. Values with different letters are significantly different from each other

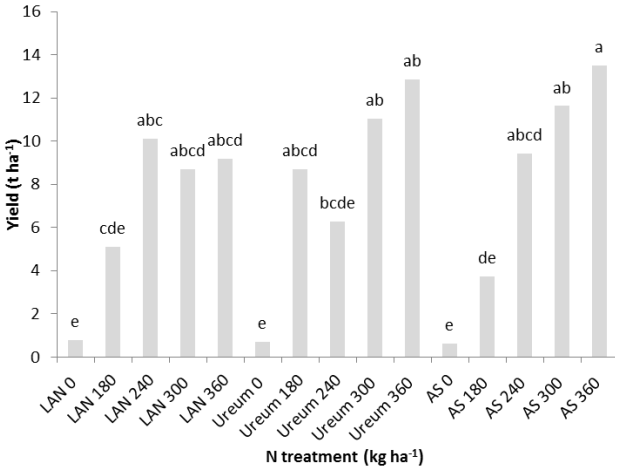

Fig. 1: Influence of N fertilizer types (AS = Ammonium sulphate) on the yield for the total harvest calculated in t ha^{-1} for *S. frutescens* (LSD 0.05 =5.769). Values with different letters are significantly different from each other

yield of the control of 15 t ha^{-1}, although not statistically different (Fig. 2). Only the plots that received high P treatments for *S. frutescens* survived, with a yield recorded and subsequently no statistical analysis could be performed. The only treatments which resulted in a yield was 100 kg P ha^{-1} superphosphate (1.1 t ha^{-1}), 120 kg P ha^{-1}

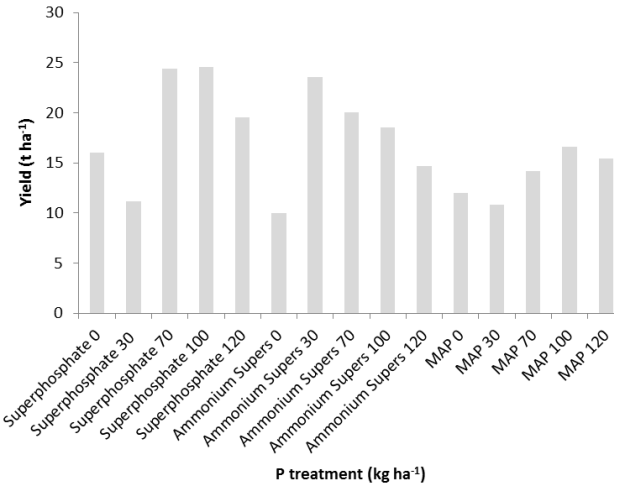

Fig 2: Influence of P fertilizer types on the yield for the total harvest calculated in t ha^{-1} for *L. leonurus*. No significant differences were obtained

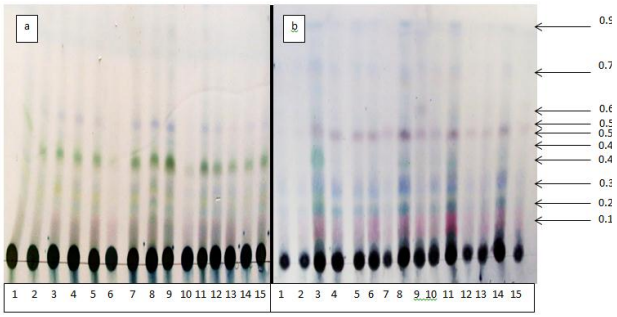

Fig. 3: Thin layer chromatography analysis of *S. frutescens* methanol extracts (a) and ethyl acetate extracts (b) with the corresponding Rf values

superphosphate (1.1 t ha^{-1}), 120 kg P ha^{-1} ammonium supers (1 t ha^{-1}), 100 kg P ha^{-1} MAP (1.2 t ha^{-1}) and 120 kg P ha^{-1} MAP (1 t ha^{-1}). The N treatments resulted in a significant increase in yield, with very low or no increases obtained for the P fertilizer trials for both species. Various differences within the chemical composition were observed with the TLC analysis of various extracts.

Chromatographic analysis for the N trial for *S. frutescens* in Fig. 3 revealed various differences in the profile for the plants amended with different N sources and levels (Fig. 3). There is however no pattern where a specific N source increased a specific compound or an increase or decrease of specific compounds with the various N Levels.

Discussion

The study investigated the effects of three inorganic N and three inorganic P fertilizers at different levels on the growth and chemical composition of the two medicinal plants *L. leonurus* and *S. frutescens*. The field trials were conducted to determine the fertilizer requirements of the plants, without negatively affecting the chemical composition.

In this study, a relatively constant yield was obtained for the controls (treatments, 1, 6 and 11) for both species, with very low yields indicating the positive effect of the fertilizers in general on the yield. All the treatments showed significant increases in the yield for the N trial when compared to the controls, with the highest yield with LAN applied at 360 kg N ha^{-1} for *L. leonurus* at 42.17 t ha^{-1} and for *S. frutescens* the highest yield of 13.51 t ha^{-1} with the application of ammonium sulphate at 360 kg N ha^{-1}. For *L. leonurus*, the saturation point has been reached with all three fertilizers, but for *S. frutescens* there was still an almost linear increase with ammonium sulphate, even at the highest level of 360 kg N ha^{-1}. When comparing the highest yield for the two species, it is evident that *L. leonurus* is a much faster grower with a three-fold increase in yield from the control value of 13.54 t ha^{-1}. *S. frutescens*, even though a slow grower achieved a 17-fold increase from the 0.77 t ha^{-1} for the control to the highest yield which indicates the significant effect of the N fertilizer amendment on the yield of this species. It is however evident that the plant reach much lower yields when compared to *L. leonurus* at 42.17 t ha^{-1}.

For *L. leonurus*, no significant differences were obtained with the P trial, although an increase was observed for some treatments with a reasonable yield of 24.56 t ha^{-1} obtained at 100 kg P ha^{-1} applied as superphosphate when compared to the control at 15 t ha^{-1}. It therefore seems that the P requirements for this plant have been met and no further increase will be achieved by applying more P. No statistical significant differences were obtained with the P trial for *S. frutescens* as very few of the plants survived. This is therefore an indication that the plant requirement for P is very low and no increase in P could increase the survival of the plants as other elements are also needed for growth and development.

In this study, TLC analyses were performed to provide an overview of the changes in the chemical profile of the plant under different soil amendments and levels. The importance of the solvent is also indicated as the components extracted by each solvent differs and the concentration of the components also varies. The compound at Rf 0.45 is more soluble in methanol and is present in much higher concentrations than in the ethyl acetate extracts and support the selection of appropriate solvents to target specific compounds in the plant. In *S. frutescens*, no major changes could be observed in the chemical composition, but in some treatments a change in the concentration of some of the components was observed. These can be found at Rf values 0.27, 0.33, 0.47, 0.52, 0.62, 0.76 and 0.91. The changes are not consistent within the application of N source or application level. The changes are therefore random and might be attributed to primary metabolism rather than secondary metabolites. The changes in the chemical profile should however raise some concern as this might have a pronounced effect on the medicinal properties of the plants related to fertilizer type and level. Biological assays would however be necessary to determine the influence of fertilizer on the primary and secondary metabolite profile, and subsequent medicinal properties of the plants. Furthermore, extensive chemical identification of these plants is however imperative, to identify the main compounds which can be linked to the medicinal properties of the plants. In a study on *Rhodiola sachalinensis* where the active ingredient salidroside was found to be increased with rich organic matter, low pH and high levels of exchangeable nitrogen and total nitrogen, but an increase in P and K, resulted in a decrease in yield (Yan *et al.,* 2004). Even though salidroside is a phenolic compound, other elements such as iron have also shown that it can increase phenolic compounds (Dixon and Paiva, 1995) and further complicates the interpretation and decision on fertilizer application. The compound at Rf 0.62 in the ethyl acetate extract is higher in some treatments, even though the rest of the compounds in the extract are not visibly higher and is evident of a compound elevated in a certain condition as created by the treatment. This technique is, however, not quantitative and since there was no complete absence of components, a more quantitative technique should be used to identify the changes and to identify the compounds in the extracts. Both these plants are however used to treat a number of ailments, and specific bioassays should be used to determine if these qualitative differences impact on the medicinal properties of the plants.

Conclusion

N and P soil amendment significantly affected the yield of both *L. leonurus* and *S. frutescens*. Since these plants are used for various medicinal purposes, the stability of the chemical profile is of utmost importance to retain medicinal activity and changes as observed in TLC might affect it negatively. Bioassays directed at specific uses should be further investigated as soil amendment is significantly affecting the growth and development of both plants and resulted in changes in chemical profile and concentration. Extensive chemical identification is also important to determine the effect of soil amendment on specific compounds, especially compounds responsible for medicinal properties.

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