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Full Length Research Paper

Effect of $AgNO_3$ on androgenesis of *Brassica oleracea* L. anthers cultivated *in vitro*

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The present article is a synthesis study accomplished at Vegetable Research and Development Station Bacau regarding the implication of silver nitrate ($AgNO_3$) in modulating the morphogenetic reaction of white cabbage anthers cultivated *in vitro*. According to literature, silver nitrate is a strong inhibitor of ethylene action. Embryo formation, shoot generation and rooting of plantlets are key points on which relies the entire concept of *in vitro* tissue culture. Silver ions, in the form of nitrate play an important role in promoting the somatic embryogenesis and organogenesis, which led its wide spread use in different plant tissue culture *in vitro*. Therefore, our researches focused on testing different concentrations of $AgNO_3$ (5, 10, 15, 25, 30, 50 and 60 µM) added to a pre-tested medium formula– Murashige and Skoog (MS), 1962 basal medium supplemented with benzylaminopurine (BAP)- 8.9 µM and naphthylacetic acid (NAA) 2.7 µM. The biological material is represented by unopened flower buds containing anthers with microspores at late uninucleate to binucleate stage. In the culture condition investigated in this study, the data obtained promotes the utilization of $AgNO_3$ in a concentration of 50 µM for the successfully induction and sustaining of regenerative processes of white cabbage anthers cultivated *in vitro*.

Key words: Buds, embryogenesis, ethylene, organogenesis, regeneration.

INTRODUCTION

Ethylene a gaseous plant hormone produced by tissues as shown by many *in vitro* studies affects callus growth, shoot regeneration and somatic embryogenesis *in vitro.* Due to the fact that all the processes of plant cells and tissue culture *in vitro* are developed in a rather closed medium (culture recipients with limited or no gaseous exchange), the influence of ethylene materialized through abscission, senescence and growth retardation can negatively affect the success of the *in vitro* regeneration. The accumulation of this gas in the culture recipients inhibits the shoots and embryo initiation and growth, hindering the ability of regeneration of plantlets from different plant tissues (Pua and Chi, 1993). Silver nitrate is recognised in literature as one of the most effective inhibitor of ethylene action. Through its involvement in regulating the production or action of ethylene, silver nitrate proved to be an important tool in modulating, to a certain extent, the growth and development of some tissue cultures *in vitro.*

Over time, a large number of reports underlined the effectiveness of silver nitrate addition to tissue culture medium of different plants (Kumar et al., 2007). In Brassica sp., ethylene produced by explants cultivated *in vitro* was responsible for the recalcitrancy in regeneration, reported in cauliflower and Chinese cabbage (Zhang et al., 2000).

The addition of silver nitrate in tissue culture medium

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Abbreviations: BAP, Benzylaminopurine; **NAA,** naphthylacetic acid.

allowed the improvement of regeneration process among these plants. The authors obtained good results on different concentrations of silver nitrate (Anantasaran et al., 2008), therefore the present study aimed to determine the best quantity of silver nitrate that should be added to culture medium in order to support the regeneration processes in *Brassica* anthers cultivated *in vitro.* We developed a complete screening of different concentrations of $AgNO_3$, (5, 10, 15, 25, 30, 50 and 60 µM) added to a pre-tested medium formula– MS, 1962 basal medium supplemented with BAP 8.9 µM and NAA 2.7 µM, in order to determine whether and which concentration of silver nitrate can improve the regeneration system. The hormonal formula utilised was determined to be the most effective in our previous experiments.

MATERIALS AND METHODS

Plant growth conditions

In this research, two open pollinated genotypes (DL 20 and DM 56) provided by Vegetable Research and Development Station Bacau were used as donor plants. The mother plants were grown in controlled conditions in greenhouse, with proper regime of fertilisation, irrigation and phyto-sanitary control. The biologic material is represented by unopened flower buds at 3.0 to 3.4 mm length which contain anthers with microspores at late uninucleate to binucleate stage. The developmental stage of the microspores was determined through observation under microscope according to 1% aceto-carmine method.

Sterilization

The excised buds were surface sterilized in 0.1% mercuric chloride (w/v) for 15 min, followed by rinsing in sterile distilled water for 3 to 4 times. After sterilization, the buds were dissected, the anther filaments removed and under aseptic conditions, the anthers were inoculated in sterile tubes on basal MS (Murashige-Skoog, 1962) nutrient medium supplemented with BAP 8.9 µM and NAA 2.7 µM. Starting with this basic mineral and hormonal medium, we tested 8 concentrations of $AgNO_3$ as follows: V1- control without silver nitrate, V2- 5 µM, V3- 10 µM, V4- 15 µM, V5- 25 µM, V6- 30 µM, V7- 50 µM and V8- 60 µM.

The pH was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved at 121°C (1.06 kg/cm^2) for 25 min.

Culture techniques

The cultures were incubated at a temperature of 33°C for one week in complete dark condition, followed by their transfer in culture chambers with controlled light, humidity and temperature control at 25°C, a 16-h photoperiod and 5000 lx light intensity. Four to five weeks after inoculation of anthers, they were removed aseptically from the culture tubes on a sterilized glass plate inside the laminar airflow cabinet and were placed again on freshly prepared sterilized medium with the same formula. The sub cultured culture tubes were then incubated at 25°C with 16 h photoperiod for 5 to 7 days. Repeated sub cultures were done at an interval of 30 days and incubated under the same temperature as mentioned previously. The culture vessels showing signs of contamination were discarded. Day to day observation was carried out to note the responses.

Rooting and acclimatization

After 3 to 4 weeks, when regenerated shoots reached a length of more than 4.0 cm, they were separated and transferred to MS basal medium supplemented with 2.7 µM NAA for rooting. The rooted plantlets were transferred to the hydroponics conditions in bottles and hardened by maintaining a high humidity (90% RH) during the first week of hardening, which was gradually decreased and it resulted in more than 95% survival of plantlets.

Statistical analysis

Ten anthers per sterile tube, in three replications were utilized for each variant. The percentage of anthers forming regeneration structures and the mean number of shoots per explant was recorded. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan multiple comparison test at $P < 0.05$.

RESULTS AND DISCUSSION

The regeneration structures got initiated after 14 to 20 days of cultures. The anthers started to grow in size and gradually we observed the initiation of callus, regeneration of shoot from callus or directly from anthers.

The results obtained suggest that the poor regeneration response found in the control variant (without $AgNO_3$, variant V1) may be associated with ethylene production by the *in vitro* cultured cells or tissues.

The increasing enhancement of reactive anthers (Figure 1) of both cabbage genotypes, obtained in variants where $AgNO_3$ was added in different concentrations suggests that until a certain level, the addition of silver nitrate inhibits the ethylene production, acting as a promoter of regeneration processes in *Brassica* anthers. Our results supports the theory of Zhang et al. (1998) which suggested that the increase of shoot regeneration frequency registered on cotyledonary explants cultivated on media supplemented with $AgNO_3$ is due to the intervention of silver nitrate as a suppressor of an ethylene signal transduction pathway.

When compared with the control variant, the anthers inoculated on silver nitrate containing mediums started to turn green, improved in volume gradually and reacting towards the callus formation or direct organogenesis.

The effect of $AgNO_3$ on the number of anthers that generated callus

The anthers generated small calluses, mainly on the filament side of the anthers in two to three weeks after inoculation. The number of calluses, type and consistency were observed both on control group (anthers

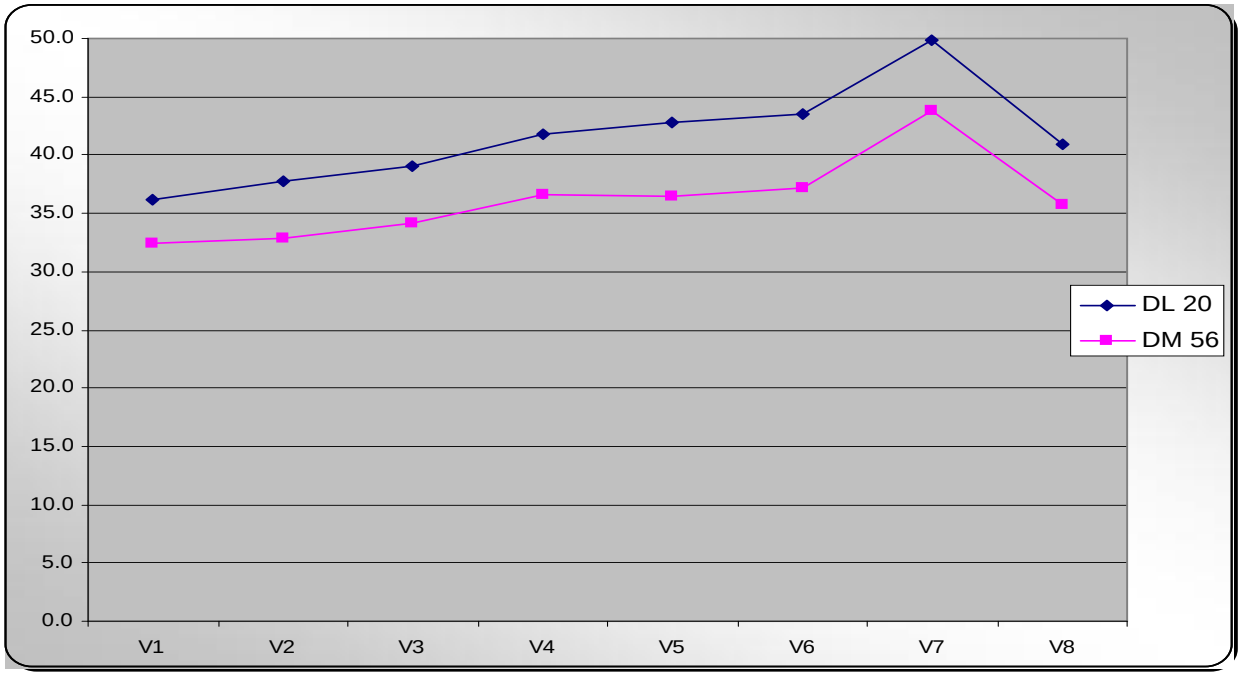

Figure 1. The influence of $AgNO_3$ on the number of anthers that reacted either towards callus or shoot formation. The data are presented as percentage of the total number of inoculated explants.

cultivated on medium without silver nitrate) and on $AgNO_3$ applied mediums (variants V2 to V8).

As shown in Table 1, the addition of silver nitrate improved the ability of *Brassica* anthers to regenerate on MS medium supplemented with BAP 8.9 µM and NAA 2.7 µM. On all variants, the results are higher when compared with the control (variant V1) where no silver nitrate was added. The results are presented as percentage of the total reactive explants.

The cytological observations of callus underlined the two type of callus: Embryogenic and nonembryogenic. The embryogenic callus, with a relatively undifferentiated structure had isodiametric cells and areas with somatic embryos in different stages of development (Figure 2). The nonembryogenic callus had large, anisodiametric cells, with tracheids but no embryogenic areas. The colour of the callus varied between white green (Figure 3) to dark green (Figure 4) and brown. For both genotypes, the highest frequency of callus formation (56.0% for genotype DL20 and 58.8% for DM56 genotype) was observed in variant V7 with 50 µM $AgNO_3$, while the least (32.5%, respectively 29.8%) was obtained in the control variant.

By transferring the callus to fresh medium, the regeneration processes evolve, allowing the appearance of shoots. The shoots aseptically removed from tubes and placed on new fresh media allowed the continuation of regeneration processes through the appearance of adventitious shoots at the base of the newly formed ones (Figure 5).

The effect of $AgNO_3$ on the number of anthers that generated shoots

Enhancement of adventitious shoot formation by $AgNO_3$ addition to the culture medium has been reported also by other authors. Williams et al. (1990) reported in *Brassica oleracea* var. *gemmifera* the enhancement of shoot regeneration from callus on low hormone medium supplemented with $AgNO_3$. The same good results were obtained also for other species: Akasaka-Kennedy et al. (2005) with rapeseed, Misra and Datta, (2001) with white marrygold, Buyukalaca et al. (2004) with pepper anthers, Anantasaran et al. (2008) with *Zinnia* sp., Yupaporn and Sompong, (2012) with *Hevea* sp., Naik and Chand, (2003) with pomegranate, etc.

The results obtained during the present study show that the addition of silver nitrate improved the capacity of direct organogenesis on all variants when compared with the control. When compared with the control variant (V1 with no silver nitrate addition), on MS medium supplemented with $AgNO_3$, multiple shoots were observed directly from anther tissue, within 3 to 4 weeks after inoculation. The first visible change in anthers was enlargement in size within the first week after inoculation.

Table 1. Effect of different concentrations of silver nitrate on callus formation of *Brassica oleracea* L. anthers.

Variant	Genotype DL20			Genotype DM 56		
	Anthers generating callus (%)	Callus type	Fresh biomass (g)	Anthers generating callus (%)	Callus type	Fresh biomass (g)
V1	32.5	Nonembryogenic, brownish	1.80	29.8	Nonembryogenic, white green	2.12
V2	38.6	Nonembryogenic, yellow green	2.20	33.1	Nonembryogenic, white green	2.10
V3	47.1	Embryogenic, white green	1.29	39.6	Embryogenic, white green	0.43
V4	50.6	Embryogenic, green	0.89	42.9	Embryogenic, green	0.44
V5	51.3	Embryogenic, green	0.57	46.6	Embryogenic, dark green	0.61
V6	54.1	Embryogenic, dark green	0.88	50.9	Embryogenic, dark green	0.78
V7	56.0	Embryogenic, dark green	0.95	58.8	Embryogenic, dark green	0.49
V8	50.3	Embryogenic, green	1.29	55.2	Embryogenic, yellow green	0.44

Adventitious shoots developed directly from the anther's surface and initially appeared as small outgrowths (Figure 6) that developed gradually in shoots. At the base of the initial shoot, new shoots with one or two branches started to appear, which allowed the continuation of the regeneration process. Among the seven $AgNO_3$ concentrations, 30 and 50 µM, added to medium (variant V6 and V7), initiated significantly better response in relation to callus formation and shoot regeneration and induced higher number of shoots per explant in both genotypes.

Regeneration percentage was affected by silver nitrate concentration (Table 2). Of the various levels of $AgNO_3$ tested, the variant with 50 µM proved to be most effective, as on this medium, not only the number of shoots per explant was maximal but also, the number of explants producing multiple shoots was the highest. On lower concentration of silver nitrate from 5 to 25 µM, the number of shoots per culture was reduced. Similarly, in higher concentrations of $AgNO_3$ (60 µM $AgNO_3$), the number as well as the length of shoots similarly reduced the concentration of 50 µM added to MS medium supplemented with BAP 8.9 µM and NAA 2.7 µM increased the percentage of organogenic anthers at 42.9%- DL 20 genotype, respectively 40.1%- DM 56 genotype from the total number of reactive anthers, when compared with 26.4, respectively, 21.1% obtained in control variant. The same concentration was found to be effective in cotyledon and hypocotyl explants of cucumber (Mohiuddin et al., 1997), on single node stem section explants of potato (Hakan, 2004), while other authors reported good results either on lower concentrations: 41 µM with gloxinia (Park et al., 2012) 5.8 µM with pepper (Aboshama, 2011) or higher concentrations: 60 µM with turnip (Takasaki et al., 2004), 88 µM with pepper anther (Taskin et al., 2011). These results suggest that the concentration of silver nitrate is strongly dependent on species; further studies need to be done in order to meet the requirement of each species, as its presence in culture medium was found to be highly effective in enhancing the callus growth, shoot regeneration and somatic embryogenesis in most of the studied species (Kumar et al., 2009).

After 3 to 4 weeks, when regenerated shoots reached a length of more than 4.0 cm, they were separated and transferred to MS basal medium supplemented with 2.7 µM NAA for rooting. The rooted plantlets were transferred to the hydroponics conditions in bottles and hardened by maintaining a high humidity (90% RH) during the first week of hardening, which was gradually decreased and it resulted in more than 95% survival of plantlets.

Conclusions

Silver nitrate can be successfully utilized for improving the morphogenetic reaction of white cabbage anthers. In the conditions tested in our study (basal medium, genotype, temperature, etc), the optimum concentration of silver nitrate proved to be 50 µM. Due to the fact that many

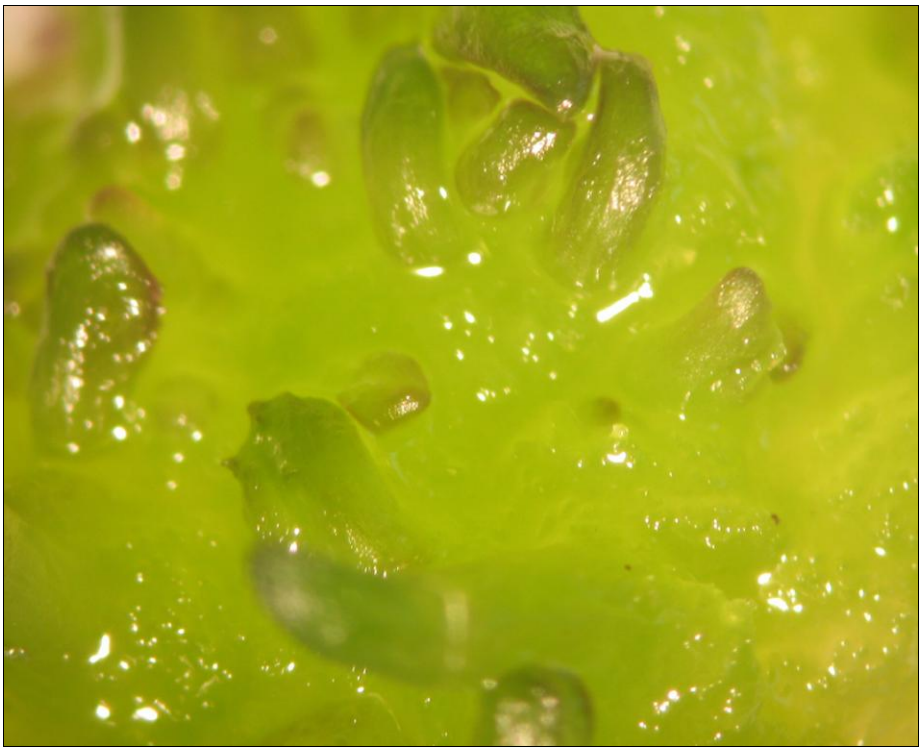

Figure 2. Somatic embryos in different stages of development.

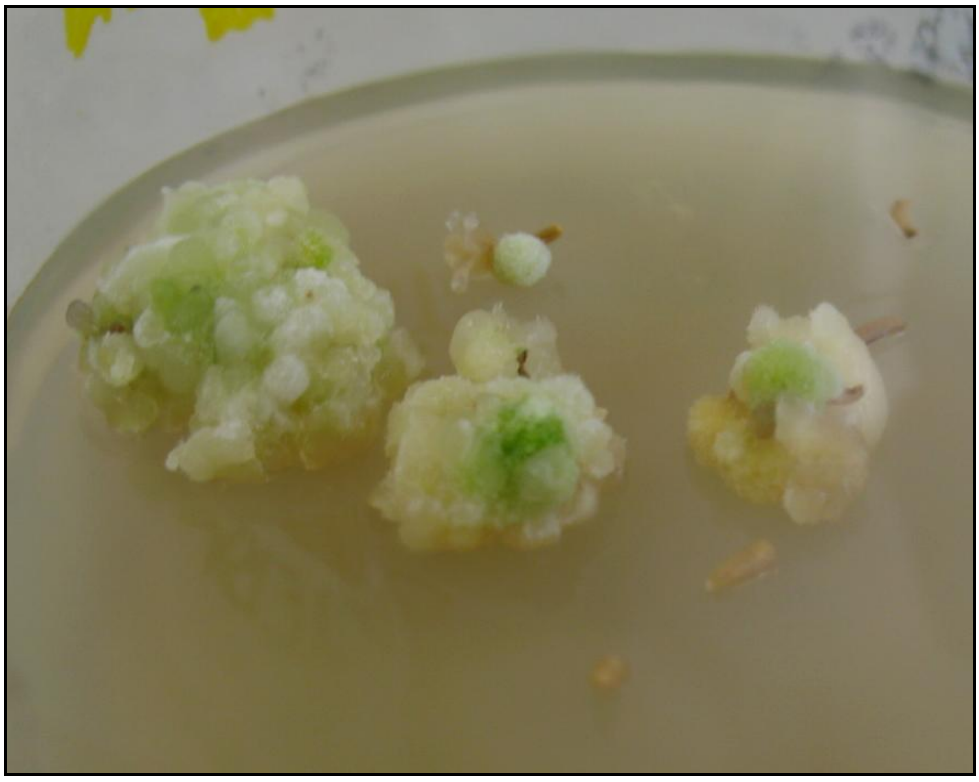

Figure 3. White green callus generated from anther generated from anther tissue.

Figure 4. Compact, globular and green coloured callus.

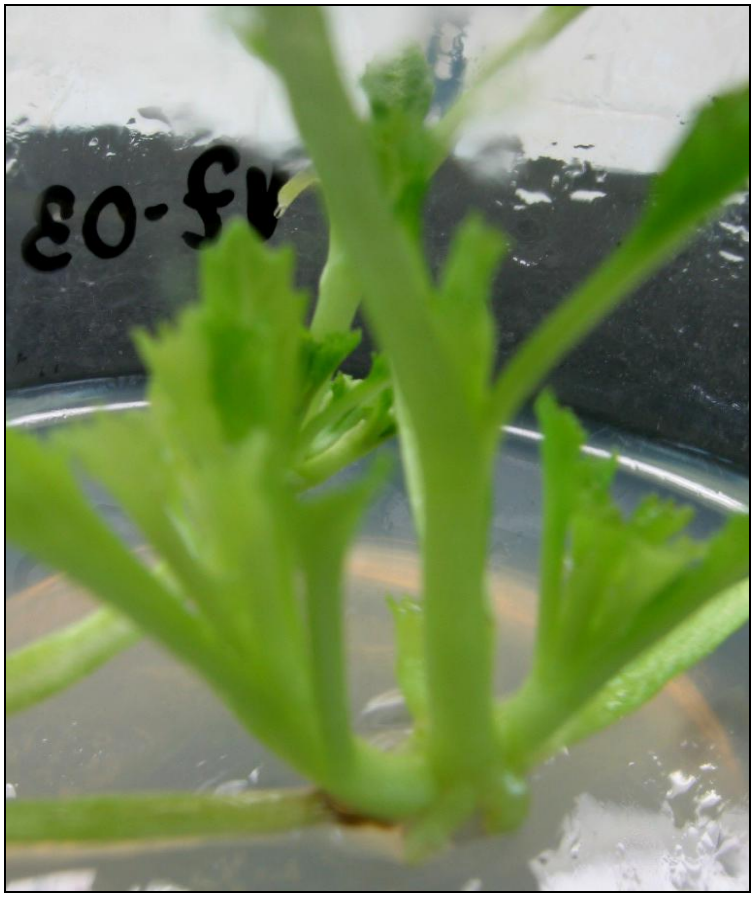

Figure 5. Formation of adventitious shoots at the base of newly transferred microplants.

Figure 6. Small outgrowths that evolved directly from anther tissue (foto stereomicroscope).

Table 2. Effect of different concentrations of silver nitrate on organogenesis of *Brassica oleracea* L. anthers after 60 days (3 subcultures) – means ±SE.

Variant	Genotype DL20			Genotype DM 56		
	No of anthers generating shoots (%)	Average number of shoot/explant	Average length of shoots	No of anthers generating shoots (%)	Average number of shoot/explant	Average length of shoots
V1	26.4	24±0.18	3.9±0.28	21.1	22±0.15	3.4±0.24
V2	30.3	25±0.32	4.1±0.37	23.8	33±0.72	3.6±0.20
V3	31.8	34±0.36	3.2±0.28	25.3	32±0.24	4.4±0.10
V4	38.3	34±0.52	4.5±0.39	26.9	41±0.88	4.2±0.15
V5	37.6	36±0.48	4.8±0.52	32.3	43±0.85	4.3±0.36
V6	41.8	41±0.35	4.8±0.46	38	45±0.65	4.6±0.41
V7	42.9	47±0.28	4.5±0.38	40.1	46±0.38	4.2±0.38
V8	38.6	36±0.18	4.3±0.35	33.7	33±0.25	3.5±0.24

other factors might be genotypic dependence, further work should be done in order to determine the appropriate concentration for each specie or genotype.

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