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MICROPROPAGATION OF MATURE CORK-OAK (*QUERCUS SUBER* L.) : ESTABLISHMENT PROBLEMS

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RESUM

S'han desenvolupat diversos mètodes per a l'estandardització de la fase d'establiment durant la micropropagació de la surera (*Quercus suber* L.) adulta. Gemes axil.lars i terminals, cultivades en medi de Gresshof y Doy (1972) (GD), van ser utilitzades com a primer explant. L'establiment dels cultius va ser molt dificultós per causa de l'elevat ennegriment dels teixits y/o del medi i per l'elevada contaminació bacteriana. Els problemes d'ennegriment, deguts probablement a l'exudació de compostos fenòlics, eren més importants a l'hivern. No obstant, es va aconsenguir l'establiment dels cultius al llarg de tot el cicle anyal, presumiblement pel precondicionament dels esqueixos. Els explants es van establir en medi GD adicionat de 1 mgl^{-1} de 6-benzilaminopurina (BAP).Cada 4 setmanes es procedia a efectuar un subcultiu en el mateix medi GD i a induir la proliferació amb una taxa de multiplicació de 4:1. S'induïa l'elongació dels brots mitjançant una disminució progressiva de la concentració de BAP. Els millors resultats per a l'arrelament *in vitro* es van aconseguir sobre medi en agar solidificat adicionat amb 1 mgl^{-1} d'àcid indolacètic (IAA). També es va assajar el medi líquid (sistema sorbarod) i l'arrelament *in vivo*.

ABSTRACT

Procedures have been developed to standardize the establishment stage during mature cork-oak (*Quercus suber* L.) micropropagation. Axillary and terminal buds cultured in Gresshof and Doy (1972) (GD) medium were used as first explant. Establishment of cultures was very difficult due to browning of the tissue and/or the medium and bacterial contamination. Browning problems, probably due to phenolic compounds exudation of the primary explant, were found to be higher in winter. Nevertheless, initiation of cultures was possible all over the year, presumably due to the preconditioning of cuttings. Explants were established in a GD medium containing 6-benzylaminopurine (BAP) 1 mgl^{-1}. Every 4 weeks the cultures were subcultured to the same GD medium and induced to proliferate being 4:1 the multiplication rate. Shoots were induced to elongate by decreasing BAP concentration. *In vitro* rooting on agar-solidified medium supplemented with 1 mgl^{-1} indolacetic acid (IAA) gave the best results. Liquid medium (sorbarod system) and *in vivo* rooting were also assayed.

Key words: browning, contaminants, cork-oak, micropropagation, phenolic compounds, *Quercus suber* L., tissue culture.

INTRODUCTION

Quercus suber L., the primary source of industrial cork, occurs naturally and is cultivated in the western Mediterranean region. Cork-oak is essentially seed-propagated but as hibridization occurs freely, seed-raised plants present great variability and a high percentage of undesirable phenotypes. Production of the best

cork-oak cultivars involves the selection of superior genotypes which are then propagated to ensure uniformity of propagules.

The mass clonal propagation of selected genotypes is a potentially valuable method for accelerating the improvement of this important species since conventional asexual methods have been unsuccessful. The number of papers on *in vitro* regeneration of woody species has risen drastically in recent years and different difficulties have been reported when field material was used. However there have been few reports concerning tissue culture of *Q. suber*. The first report was made by Jacquiot (1952), who obtained callus from *in vitro* cultures of cambial tissue. Bellarosa (1981) obtained subcultures of axillary shoots from *in vitro* cultures of cork-oak embryos and Pardos (1981) has also produced cork-oak cultures from nodal segments of 12 month-old seedlings. In both works little success was achieved at the rooting stage. In 1987 Maâtaoui and Espagnac reported preliminary results on neoformation of somatic embryo-like structures on *Q. suber* and in 1988 Deidda *et al.* reported plantlet regeneration from axillary shoots of seedlings. All these reports have concerned seedling material.

Although micropropagation from adult tissues is desirable to achieve and maintain genetic gains, results dealing with mature tissues are still limited and scarce. Recently, Manzanera and Pardos (1990) reported *in vitro* plantlet multiplication, of both cork-oak seedlings and stump-sprouts material. Although these authors had no problems with contaminants, high percentage of toxicity was observed originating large material losses. The initial step in the micropropagation process is to obtain aseptic cultures of the selected plant material. This objective is usually difficult to achieve when field material is used. Success at this stage firstly requires that explants should be safely transferred to the culture environment and secondly that there should be an apropriate reaction (George and Sherrington, 1984).

Contamination poses one of the greatest problems in the micropropagation of this species, a problem made even more acute by the browning increase after the use of sterilizing agents. Consequently, it is necessary to develop standard procedures for culture initiation of adult material when the goal is mass clonal propagation.

For the research work here described, we investigated the micropropagation of the cork oak from adult material with special attention to establishment problems, namely contaminants and browning of the tissue and/or the medium. The seasonal effect of the sampling time and conditioning of field material were also investigated.

MATERIALS AND METHODS

Hardwood stem cuttings of mature trees, 40 cm long, were collected all over the year. They were defoliated and stored at 5°C in the dark. Prior to use a surface-sterilization was done in a NaOCl (20%)(v/v) and teepol (5%)(v/v) solution for 30 min, followed by immersion in 3 gl^{-1} benlate for 1 hour. After this previous sterilization the cuttings were induced to sprout under controlled conditions in order to make a preconditioning of plant material. The cuttings were maintained wet in a 80% humidity chamber at 24°C, under a 16 h photoperiod at 60 $\mu molm^{-2}s^{-1}$ produced from cool-white fluorescent lights (Philips TLD 18 W/33). After two weeks sprouts were long enough to be defoliated. Axillary and terminal buds were used as primary explants. In order to control contaminants they have been carefully sterilized. Different sterilizing

procedures were assayed: **1-** Soaking for 15 min in a 5% (v/v) sodium hypochlorite solution (available chlorine 5%) containing 0.01% (v/v) teepol. **2-** Soaking for 15 min in a 5% (w/v) calcium hypochlorite solution (freshly prepared and filtered) containing 0.01% (v/v) teepol. **3-** Soaking for 15 min in a 5% (w/v) cetrimide solution containing 0.01% (v/v) teepol. **4-** Soaking for 1 min in a 0.1% (w/v) $HgCl_2$ solution followed by procedure 1.

All treatments were followed by three rinses with sterilized destilled water prior to culture in the media. GD (Gresshof and Doy, 1972) containing 20 gl^{-1} sucrose and gelled with 7 gl^{-1} agar (bacto DIFCO) was used as culture medium. The pH was adjusted to 5.8 before autoclaving. The explants were placed on 30 ml of medium in 200 ml glass jars stoppered with polyethylene caps, previously sterilized at 121°C for 20 min. The cultures were maintained at 25°C, under a 16 h photoperiod at 60 $\mu molm^{-2}s^{-1}$ produced from cool-white fluorescent lights (Phillips TLD 18 W/33). Establishment was carried out during 4 weeks in GD medium. Different concentrations of 6-benzylaminopurine (BAP) (0.1, 0.5, 1, 1.5, and 2 mgl^{-1}) were assayed.

After two weeks the percentage of contaminated explants was registered. According to some authors (George and Sherrington, 1984) this period is sufficient for the appearance of most contaminants.

Seasonal effects of the sampling time on browning incidence was researched. For this purpose cuttings were collected throughout the year.

Different experiments were assayed to control or prevent browning of the medium and/or the tissue, usually ascribed to oxidation of phenols. These involved the addition of antioxidants to the medium, dark incubation of cultures, different salt concentrations and addition of BAP. The following assays were studied, according to a control: **1)** Control: GD medium without growth regulators. **2)** Murashige and Skoog (1962) medium (MS) without growth regulators. **3)** Control + polyvinylpyrrolidone (PVP) 1 gl^{-1}. **4)** Control + ascorbic acid 100 mgl^{-1} + citric acid 150 mgl^{-1}. **5)** Control + darkness during 24 hours. **6)** Control + darkness during 48 hours. **7)** Control + BAP 1 mgl^{-1}

After 48 hours of culture initiation the percentage of explants with a halo of brown substances was observed. At the end of establishment the percentage of viable explants was registered. «Viability» is here defined as the number of explants in active growth. The cultures were induced to proliferate in a GD medium supplemented with BAP 1mgl^{-1}.

In vitro and *in vivo* rooting were assayed. Shoots were cultured in liquid (sorbarod system) or agar solidified GD medium supplemented with IAA 1 mgl^{-1}. *In vivo* rooting was achieved by dipping the shoots' basal ends in a 4 mgl^{-1} IBA (indolebutyric acid) solution for 2 min and rooted as micro-cuttings in a peat + perlite mixture.

RESULTS AND DISCUSSION

Buds taken from mature parts of trees are reluctant to grow *in vitro*. Seasonal factors may reinforce natural dormancy in buds from any source, so that cultures can only be readily initiated from explants gathered at certain times of the year, usually at the start of, or during, active growth of the mother plant.

In the present work, establishment of cultures from dormant axillary and terminal buds of cork-oak, taken from field material, was possible all over the year. It is known

that growth, morphogenesis and rates of propagation *in vitro* can be improved by appropriate environmental and chemical pretreatment of mother plants (George and Sherrington, 1984). Actually we did not find any difficulty with culture initiation related with seasonal factors. These results are probably due to the preconditioning treatment of the cuttings including cold treatment. As observed by Diaz-Sala *et al.* (1990) on adult hazel, during cold storage endogenous levels of promotors and inhibitors change and stimulate further responses *in vivo*. After two weeks, 70% of the buds produced shoots 4 cm in length and apical dominance was not observed. At this stage the sprouts were ready to be cut for culture *in vitro*.

Different sterilizing procedures and exposure times have been used for surface treatment of field material. In fact, high percentage of contaminants is common (De Fossard *et al.*, 1977; Barghchi, 1988) and it is the most important reason for losses during *in vitro* culture of plants (Boxus and Terzi,1987).

From all the sterilizing procedures assayed, mercuric chloride followed by sodium hypoclorite (procedure 4) was the most adequate (Figure 1). Cetrimide was completely ineffective. It is important to emphasize that procedure 4 was successful not only in terms of sterilizing efficiency but also in terms of viability. In fact ,the toxicity effects of $HgCl_2$ reported by Manzanera and Pardos (1990) were not observed.

During explant excision various compounds usually referred as polyphenols (Compton and Preece, 1986) are released which, being oxidized, turn brown or black resulting in the darkening of both tissue and culture medium. The percentage of explants exudating phenolic compounds varies according to the sterilization process and the time of sampling (Figure 1). Correlation between sterilization efficiency,

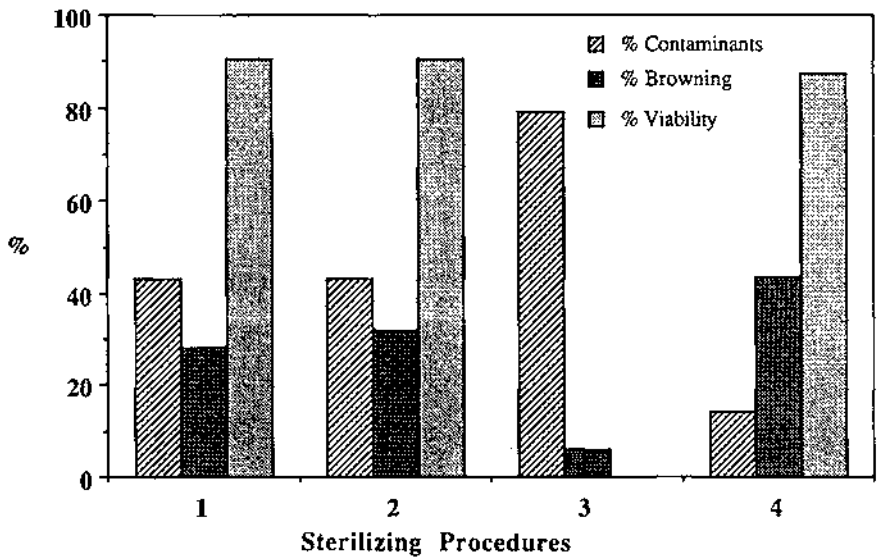

Figure 1. Percentage of contaminants, browning and viability according to different sterilization procedures. 1- Sodium hypochlorite; 2- Calcium hypochlorite; 3- Cetrimide; 4- $HgCl_2$ solution followed by sodium hypochlorite (see Materials and Methods for more details).

viability and browning incidence, during culture initiation is evident. In addition, the extent of browning increases with the efficiency of the sterilizing procedure.

Since few studies have been published describing the seasonal effect of the sampling time on browning incidence this subject was accurately studied. In *Q. suber* the percentage of browning decreases from winter to summer, being maximum in December (Figure 2). Similar results were observed in apple shoot tip explants (Hutchinson, 1982) being mid-spring or summer the best time for sampling. Also in chestnut a seasonal effect on phenolic compounds exudation was reported (Chevre *et al.*, 1983).

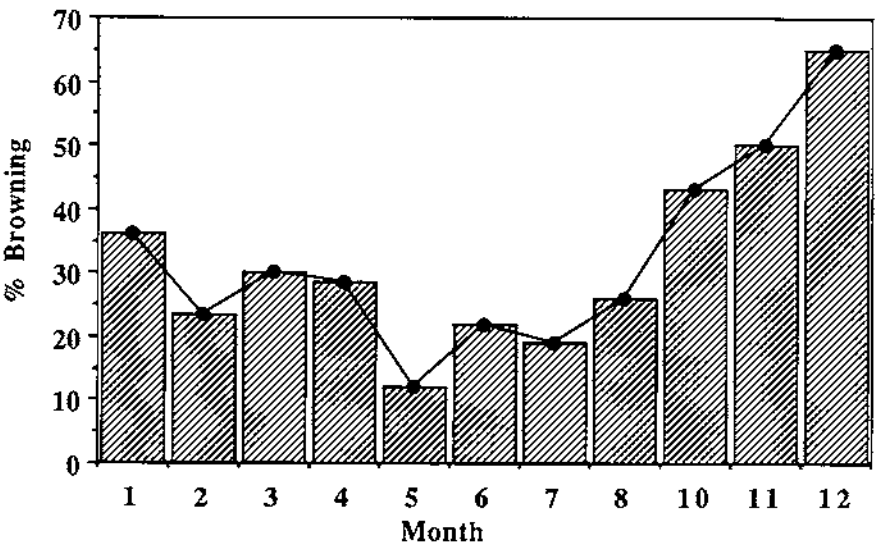

Figure 2. Effect of the sampling time, January till December 1989, on the percentage of browning.

The reduction of salt concentration in the culture medium has been stressed by some authors as being an effective mean of reducing darkening (Anderson, 1975; Gould and Murashige, 1985). According to these statements two different salt concentrations have been compared along this work: a high salt medium (MS) and a low concentrated one (GD). The results obtained show that browning incidence was higher when MS medium was used instead of GD (Figure 3).

The activity of enzymes concerned with both the biosynthesis and oxidation of phenols is increased by light (Davies, 1972). Tissue browning may therefore be reduced or prevented if newly explanted tissues are first cultured in the dark. Ahuja (1986) controlled the production of phenolics in *Q. robur* and *Q. petraea* by growing bud explants initially under low light intensity or on media containing PVP (50-100 mgl-1). Adams *et al.* (1979) conducted the culture initiation stage in total darkness and Christiansen and Fonnesbeck (1975) had good results with Hamamelis using PVP 1%. However PVP proved to be ineffective in preventing browning of chestnut

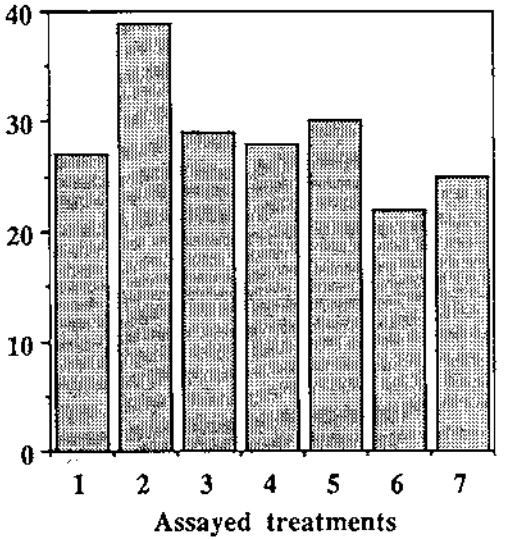

Figure 3. Effect of salt concentration, antioxidants, darkness and BAP on the browning incidence. 1- Control: GD medium; 2 - MS medium; 3 - PVP 1 gl^{-1}; 4 - ascorbic acid 100 mgl^{-1} + citric acid 150 mgl^{-1}; 5 - 24 hours darkness; 6 - 48 hours darkness; 7 - BAP 1 mgl^{-1}.

axillary bud cultures (Chevre *et al.*, 1983). In the present work neither PVP (1 mgl^{-1}) nor 24 hours of darkness reduced browning incidence, being 48 hours of darkness slightly effective (Figure 3).

Other antioxidants were tried (ascorbic and citric acid, 100 mgl^{-1} and 150 mgl^{-1}, respectively) without positive results (Fig. 3). Similar results were observed by Welsh *et al.* (1979): ascorbic acid and PVP did not control or prevent phenolic exudation into the medium or injury to buds or shoot tips of red maple.

Other authors advocate the use of a minerals-sucrose-agar medium without growth regulators for cultures initiation (Cheng, 1978; Welsh *et al.*, 1979). According to our results the addition of 1 mgl^{-1} of BAP to the culture medium does not increase browning incidence (Fig. 3), although plant growth regulators are known to have an important role in phenol oxidation.

The transfer of explants onto fresh medium 48 hours after culture initiation was used to increase explant survival as tested treatments proved to be unsuccessful. Although this methodology is very time consuming and labor intensive it was the only one which solved browning problems, as already reported for different species (Lloyd and McCown, 1980; Somers *et al.*, 1982).

The best explant establishment was achieved in a GD medium supplemented with 1 mgl^{-1} BAP, in terms of shoot length (Figure 4). Higher BAP concentrations reduced shoot growth.

After four weeks of establishment, cultures were induced to proliferate in a GD medium supplemented with 1 mgl^{-1} BAP. A proliferation rate of 4 per explant was obtained (Figure 5). Higher BAP levels resulted in good shoot proliferation but a greater percentage of the shoots were small or rosetted. The fact that this proliferation

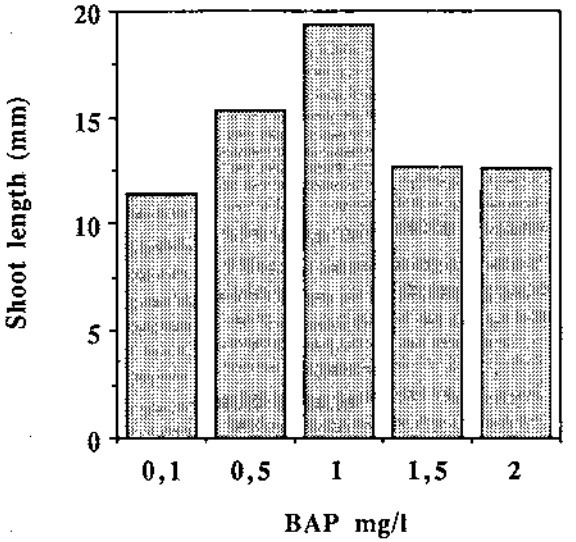

Figure 4. Effect of BAP concentration on explant length during establishment stage.

rate was higher than the one reported by Manzanera and Pardos (1990), probably reflects differences on culture medium and physiological status of the buds.

Figure 5. Shoot proliferation of *Q. suber* after 4 weeks on GD medium containing BAP 1 mgl^{-1}.

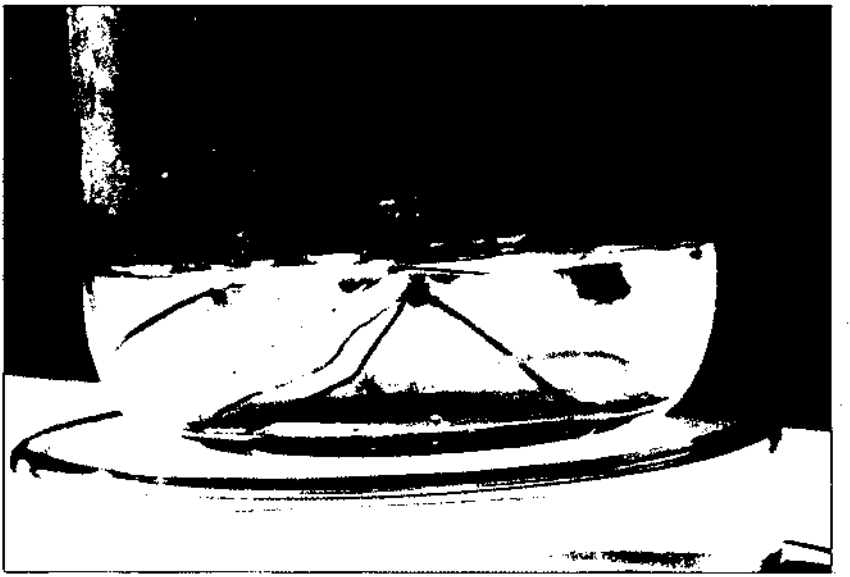

Figure 6. *In vitro* rooted shoot of *Q. suber* on agar solidified medium suplemented with IAA 1 mgl^{-1}.

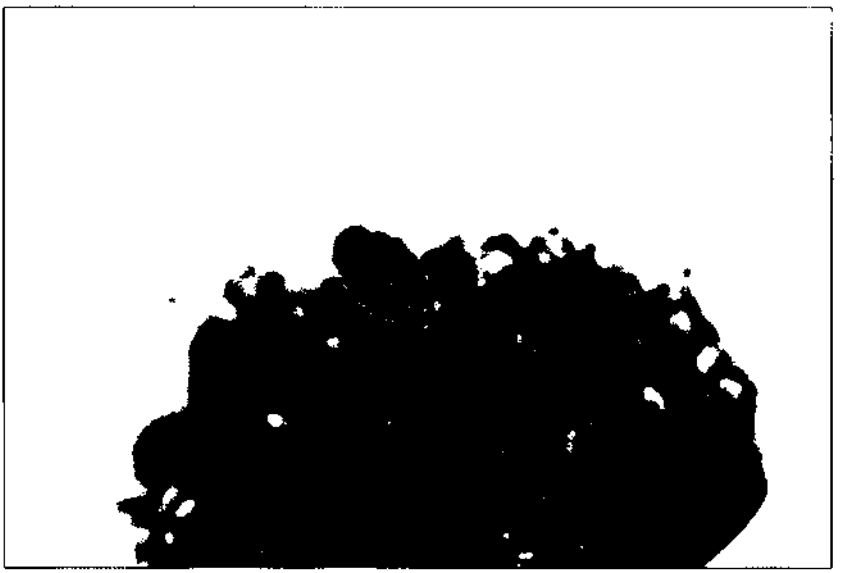

Figure.7. *In vitro*-derived plant of *Q. suber* after transfer to soil.

Every four weeks the plantlets were transferred to fresh medium and multiplication was continuously induced. Before shoot separation they were induced to elongate in a GD medium by decreasing BAP concentration.

Although some shoot lines could sporadically root on the multiplication media, the application of auxins was necessary to obtain root initiation. For the rooting experiments, shoots longer than 3 cm were separated and transferred to rooting medium. *In vitro* produced shoots rooted better on agar-solidified medium with 54% of success (Figures 6 and 7). Rooting on liquid medium was unsucessful.

CONCLUSIONS

From this work we can conclude that it is possible to initiate cultures from adult trees of *Q. suber* all over the year. The preconditioning of cuttings is probably responsible for the good reaction observed during culture establishment. Furthermore, high contamination of field material can be reduced by two sterilization steps: a first superficial sterilization of the cuttings before the preconditioning and a second strong sterilization with two sterilizing agents originating a synergistic effect on contaminants control.

Browning problems have been overcome by transferring cultures to fresh medium 48 hours after culture initiation. Although time consuming it is effective.

The proliferation rate observed (4:1) as a response to cytokinins (BAP) is very encouraging compared with previous results (Manzanera and Pardos, 1990). Proliferation and rooting rates may be considered acceptable. However new assays are under study in order to improve those rates.

The results of the study here reported constitute a promising step towards large scale *in vitro* propagation of cork-oak selected trees. Actually, the usual problems appeared at the establishment stage were overcome due to an efficient and reliable methodology.

There are other aspects which could be improved, but three objectives are worthy of mention, to which a completely optimization has not yet been found: multiplication, rooting and acclimatization.

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