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The effect of in vitro competition on shoot regeneration from hypocotyl explants of *Linum usitatissimum*

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Abstract: This study was carried out to investigate the relationship between in vitro competition and stress, and their effects on tissue culture response of *Linum usitatissimum* L. hypocotyl explants. Competition among explants was achieved by varying the spacing among the explants cultured. Four different culture spacing distances were used: 0.5, 1.0, 1.5, and 2.0 cm. Six weeks after culture initiation, hypocotyl fresh and dry weights, shoot regeneration percentage, shoot number per hypocotyl, regenerated shoot length, total shoot number per petri dish, and total chlorophyll content were recorded. The results showed that encouraging competition among explants by decreasing spacing among them from 2.0 cm to 1.0 cm increased shoot number per hypocotyl, regenerated shoot length, and total shoot number per petri dish in both cultivars. When explants were cultured at 0.5 cm spacing, significant stress-initiated decreases were observed in all parameters examined. This study showed that the success of tissue culture studies for related genotype could be increased not only by determination of correct concentrations and combinations of auxins and cytokinins in growth medium but also by evaluating competition among explants cultured.

Key words: *Linum usitatissimum*, hypocotyl, in vitro competition, shoot regeneration

In vitro rekabetin *Linum usitatissimum* hipokotil eksplantlarından sürgün rejenerasyonu üzerine etkisi

Özet: Bu çalışma, in vitro rekabet ve stress arasındaki ilişkileri ve bunların *Linum usitatissimum* L. hipokotillerinin doku kültürü tepkisine olan etkilerini araştırmak için yapılmıştır. Eksplantlar arasındaki rekabet, eksplantların kültüre alındığı mesafeler değiştirilerek sağlanmıştır. 0,5, 1,0, 1,5 ve 2,0 cm olarak dört farklı kültür mesafesi kullanılmıştır. Kültür başlangıcından 6 hafta sonra hipokotil yaş ve kuru ağırlıkları, sürgün rejenerasyon yüzdesi, hipokotil başına sürgün sayısı, rejenere sürgün uzunluğu, petride gelişen toplam sürgün sayısı ve toplam klorofil kapsamı kaydedilmiştir. Sonuçlar, eksplantlar arasındaki kültür mesafesinin 2,0 cm'den 1,0 cm'ye düşürülerek onların rekabete teşvik edilmesinin hipokotil başına sürgün sayısını, rejenere olan sürgün uzunluğunu ve petrideki toplam sürgün sayısını her iki çeşitte de artırdığını göstermiştir. Eksplantlar, 0,5 cm mesafede kültüre alındığında, stres başlamış ve bütün parametrelerde önemli düşüşler gözlenmiştir. Bu çalışma, ilgili genotip için doku kültürü çalışmalarının başarısının yalnızca büyüme ortamındaki oksin ve sitokininlerin doğru konsantrasyon ve kombinasyonlarının belirlenmesiyle değil aynı zamanda kültüre alınan eksplantlar arasındaki rekabetten yararlanılarak da artırılabileceğini göstermiştir.

Anahtar sözcükler: *Linum usitatissimum*, hipokotil, in vitro rekabet, sürgün rejenerasyonu

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Introduction

Linum usitatissimum L. is a dicotyledonous plant from the family Linaceae. It is an important source of natural fibre and industrial oil, and has the potential for meeting edible oil and protein needs (Green & Marshall, 1984). Since *L. usitatissimum* has a small nuclear genome, it has been used as a model for genetic engineering techniques (Millam et al., 1992). Moreover, *L. usitatissimum* has been used as a medicinal plant for thousands of years and components of the plant such as lignans and α-linolenic acid have been used in many drugs (Tolkachev & Zhuchenko, 2000; Spence et al., 2003).

Hypocotyl segments have been widely used in many species for in vitro regeneration (Tavano et al., 2009). It has been reported that the hypocotyl is the most suitable explant (Gamborg & Shyluk, 1976; Jordan & McHughen, 1988; McHughen et al., 1989; Dong & McHughen, 1991; Millam et al., 1992; Dong & McHughen, 1993; Yıldız, 2010) and the combination of BAP at 1 mg L^{-1} and NAA at 0.02 mg L^{-1} has been effective on adventitious shoot regeneration of *L. usitatissimum* (Dong & McHughen, 1993; Yıldız, 2010).

One of the main objectives of tissue culture studies is to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system. The introduction of foreign genes coding agronomically important traits into plant cells has no meaning unless transgenic plants are regenerated from the genetically modified cell(s). The adventitious shoot regeneration capacity of cells or tissues to be used in transformation studies affects the success of genetic transformation significantly (Jordan & McHughen, 1988; Dong & McHughen, 1993). Explant health is the main factor determining the regeneration capacity of an explant. Viability and age of the explant, and the tissue source from which the explant is excised are very important for high-frequency shoot regeneration (Yıldız & Er, 2002). Adventitious shoot regeneration subjected to gene transfer should be increased as much as possible in order to obtain a high frequency of transgenic plants. In this study, an efficient protocol was developed for increasing the adventitious shoot regeneration frequency of *L. usitatissimum* hypocotyls by utilising competition among explants cultured in vitro.

Materials and methods

Linum usitatissimum seeds of cultivars 'Ariane' and 'Verne' used in the study were obtained from Northern Crop Science Laboratories, in North Dakota, USA. Seeds were surface sterilised with 40% commercial bleach containing 5% sodium hypochlorite at 10 °C for 10-15 min with continuous stirring and then were washed 3 times with sterile distilled water at the same temperature according to the protocol described by Yıldız and Er (2002). Sterilised seeds were germinated on a basal medium containing the mineral salts and vitamins of Murashige and Skoog (MS) (1962), 3% (w/v) sucrose, and 0.7% (w/v) agar.

All cultures were incubated at 25 ± 1 °C under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16 h light/8 h dark photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl segments in 5 mm lengths were excised from 7-day-old seedlings as reported by Yıldız et al. (2003) and cultured in a petri dish at 0.5, 1.0, 1.5, and 2.0 cm spacing (Figure 1) for 6 weeks on MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.02 mg L^{-1} naphthalene acetic acid (NAA) for regeneration.

Fresh and dry weights, shoot regeneration percentage, shoot number per hypocotyl, regenerated shoot length, total shoot number per petri dish and total chlorophyll content were recorded at the end of culture. Hypocotyl segments were weighed to determine the fresh weight. The dry weight was obtained after drying explants at 105 °C for 2 h. All measurements were made using an analytical scale, with precision of 0.001 g.

Total chlorophyll content was determined in leaves of plantlets regenerated from hypocotyls according to the protocol of Curtis and Shetty (1996). Then 50 mg fresh leaf tissue was placed in 3 mL of methanol and kept in total darkness at 23 °C for 2 h. By this way, chlorophyll in fresh tissue passed through into methanol. After 2 h, absorbancies were determined at 665 and 650 nm. Total chlorophyll content was recorded as "μg chlorophyll/g fresh tissue".

Four replicates were tested. Petri dishes (100 × 10 mm) were considered the units of replication. The number of explants per replication was 11. All experiments were performed twice. Data were

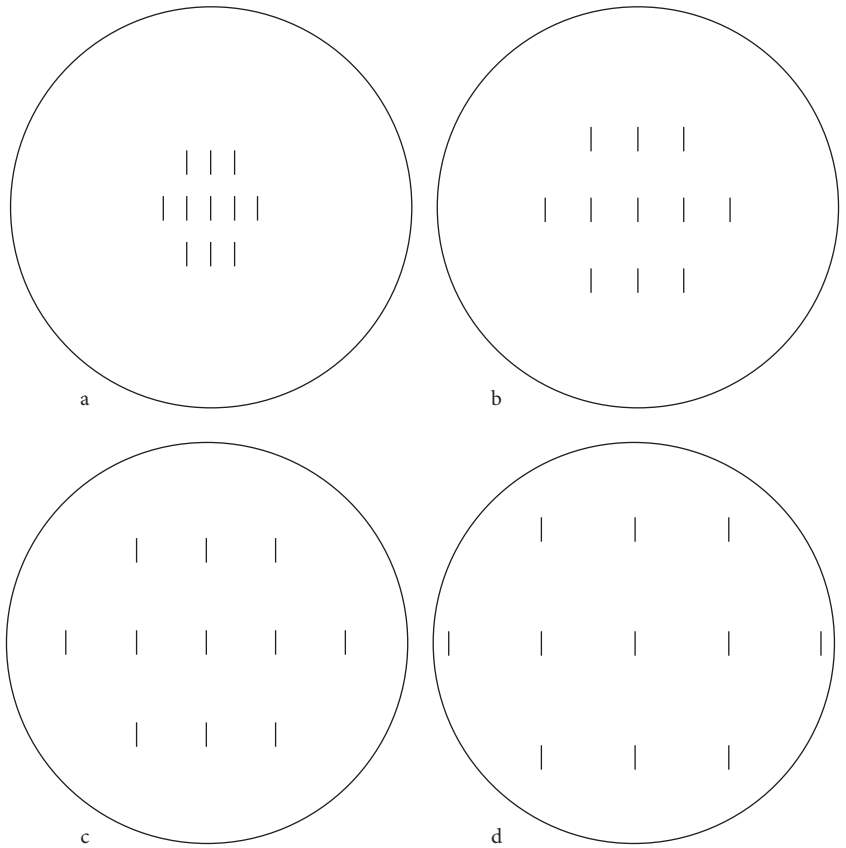

Figure 1. Schematic plan of explants cultured at different spacing in a petri dish. a- 0.5 cm, b- 1.0 cm, c- 1.5 cm, d- 2.0 cm.

statistically analysed by Duncan's multiple range test using "SPSS for Windows 15.0" (Snedecor & Cochran, 1967).

Results and discussion

Plants compete with each other in natural conditions for water, nutrients, and light. There are many studies reporting such competition among plants in field conditions (Wilson, 1988; McPhee & Aarssen, 2001). De Klerk (2007) reported that plant density as a biotic stress factor in natural conditions was one of the main reasons for competition among plants. Stoffella and Bryan (1988) reported that plant density has an effect on plant development and yield of many vegetable crops. A linear increase in fruit yield has been shown when plant density is increased (Decoteau & Graham, 1994; Jolliffe & Gaye, 1995; Morgade & Willey, 2003). Daşgan and Abak (2003) stated that early and total yield per pepper plant decreased with increased plant density, probably caused by higher interplant competition due to closer spacing. Abubaker (2008) noted that the highest planting density gave rise to the lowest yield in beans due to the high competition among plants for water and minerals. Asghari et al. (2009) reported that the chicory plant's root diameter increased for increased absoption of water under high density and high competition between plants. Although many research studies about the effect of plant density and competition have been conducted in field conditions, no such study has been reported yet under in vitro conditions. The current study was aimed at obtaining high frequency adventitious shoot regeneration by utilising in vitro competition among hypocotyls of *L. usitatissimum* (Figure 2).

Statistically significant differences were found in all parameters studied among explants of both cultivars cultured at different spacing. From the results, it was seen that fresh and dry weights of

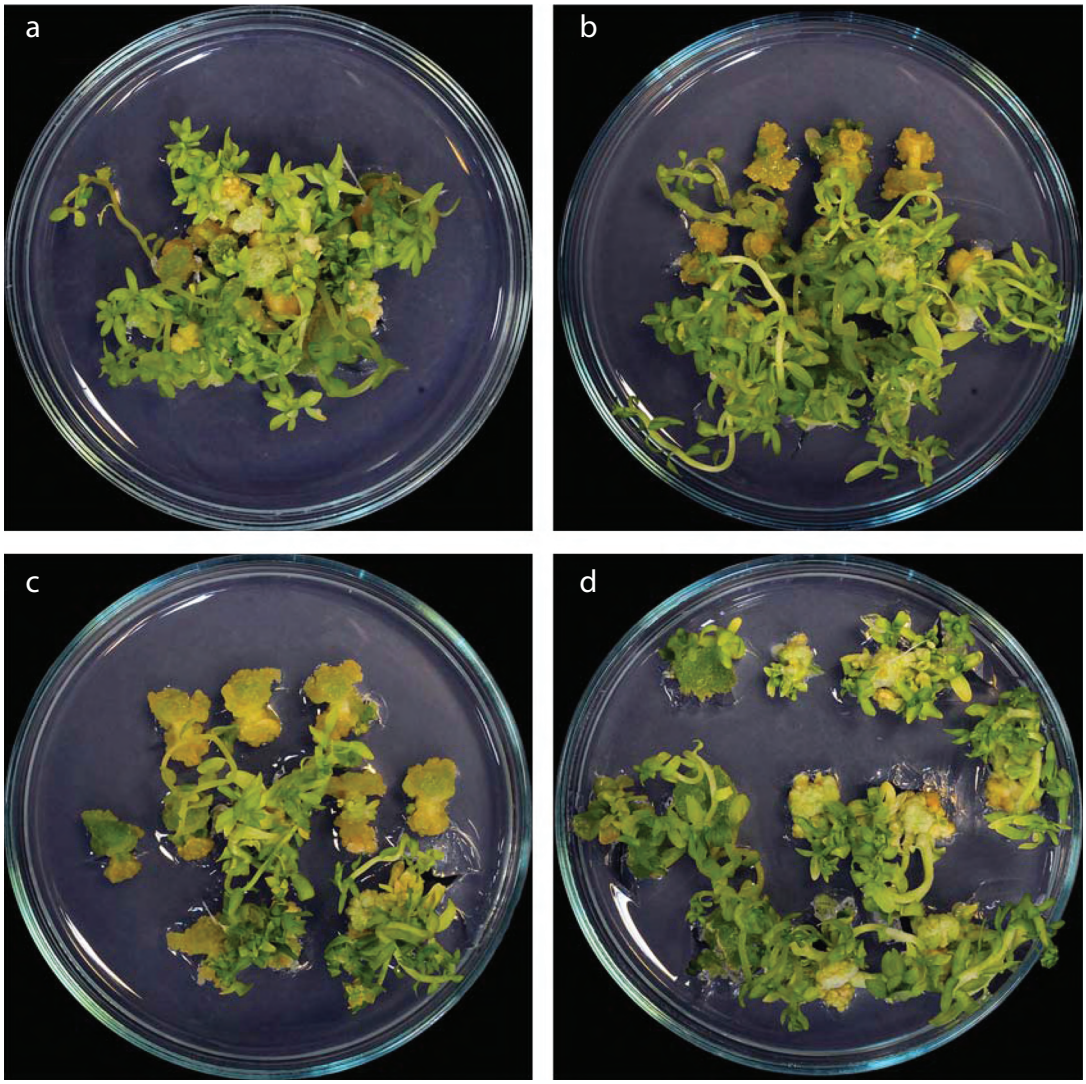

Figure 2. Adventitious shoot regeneration from hypocotyl explants cultured at different spacing (a- 0.5 cm, b- 1.0 cm, c- 1.5 cm, d- 2.0 cm) in cultivar 'Verne'.

hypocotyl explants increased with larger culture spacing. That is, the highest scores with respect to fresh and dry weights of hypocotyls were obtained at 2.0 cm culture spacing while they were lower with closer spacing (Table). These findings were parallel to the findings reported by Gersani et al. (2001) and Maina et al. (2002), who stated that plants grown alone produce more biomass or yield than those grown with others. It was clear that at the 2 cm culture spacing, the amount of water, sucrose and nutrients allocated per explant was higher than for those at lower spacing distances, which could explain the higher scores in both fresh and dry explant weights. Yıldız and Özgen (2004) reported that higher mass production in plant tissues was mainly related to an increased absorption of water and other components from the growth medium. Mills (2009) stated that sucrose in the growth medium improves biomass production. Sucrose is considered the best sugar for plant tissue culture due to its efficient uptake across the plasma membrane (Fatima et al., 2009). That is why, in our case, higher results obtained at 2.0 cm culture spacing regarding fresh and dry weights of hypocotyls could be attributed to an increased absorption of water, sucrose and nutrients from the medium as reported by Yıldız and Özgen (2004),

Table. Tissue culture response of hypocotyls of 2 *Linum usitatissimum* cultivars cultured at 4 different spacings 6 weeks after culture initiation on MS medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA

Cultivar	Spacing between hypocotyls cultured (cm)	Hypocotyl		Shoot regeneration (%)	Shoot number per hypocotyl	Regenerated shoot length (cm)	Total shoot number per petri dish	Total chlorophyll content (µg g^{-1} fresh tissue)
		Fresh weight (g)	Dry weight (g)					
Ariane	0.5	0.42 b ± 0.012	0.033 c ± 0.0012	100	2.97 b ± 0.34	3.16 b ± 0.23	32.7 b ± 3.76	224.1 d ± 12.85
	1.0	0.44 b ± 0.015	0.035 c ± 0.0009	100	4.03 a ± 0.27	4.14 a ± 0.18	44.3 a ± 2.96	332.2 b ± 5.16
	1.5	0.47 ab ± 0.039	0.042 b ± 0.0018	100	2.61 bc ± 0.22	2.92 b ± 0.25	28.7 bc ± 2.40	386.4 a ± 16.06
	2.0	0.53 a ± 0.038	0.047 a ± 0.0012	100	2.00 c ± 0.14	2.53 b ± 0.24	22.0 c ± 1.53	287.3 c ± 10.70
Verne	0.5	0.35 b ± 0.067	0.031 c ± 0.0046	100	1.85 b ± 0.11	3.48 b ± 0.53	20.3 b ± 1.20	216.7 d ± 8.62
	1.0	0.44 ab ± 0.007	0.037 bc ± 0.0015	100	3.09 a ± 0.32	5.02 a ± 0.46	34.0 a ± 3.51	326.3 b ± 11.62
	1.5	0.48 a ± 0.012	0.041 ab ± 0.0009	100	1.88 b ± 0.24	2.54 b ± 0.28	20.7 b ± 2.60	396.4 a ± 8.07
	2.0	0.56 a ± 0.020	0.047 a ± 0.0028	100	1.70 b ± 0.12	2.23 b ± 0.23	18.7 b ± 1.33	268.4 c ± 6.47

Each value is the mean of 4 replications containing 11 explants per replication. All experiments were performed twice.
Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level

Dale (1988) and Sunderland (1960). The highest fresh weight values were 0.53 g in 'Ariane' and 0.56 g in 'Verne'. These results were compatible with the ones of Asghari et al. (2009) reporting that the average total fresh weight per chicory plant was higher at the lower plant density. Dale (1988) reported that the fresh weight increase was mainly due to cell enlargement by water absorption, cell vacuolation, and turgor-driven wall expansion. On the other hand, the highest dry weight values were 0.047 g in 'Ariane' and 0.047 g in 'Verne'. Increase in dry weight was related to cell division and new material synthesis causing higher mass production (Sunderland, 1960). Deficiencies in water, sucrose and nutrients occurring at 0.5 cm culture spacing reduced fresh and dry weights of hypocotyls were reported by Yıldız and Özgen (2004).

The highest values in shoot number per hypocotyl and in regenerated shoot length were recorded at 1.0 cm spacing in both cultivars. The highest values in shoot number per hypocotyl were 4.03 in 'Ariane' and 3.09 in 'Verne' while the longest regenerated shoot lengths were 4.14 cm in 'Ariane' and 5.02 cm in 'Verne' (Table). From the results, it could be concluded that the 1.0 cm culture spacing encouraged explants to compete with each other for the constant amount of water, sucrose and nutrients in the growth medium. At 0.5 cm culture distance, significant decreases were observed in all parameters studied, which could be attributed to stress among explants caused by inadequate water, sucrose and nutrients available in the growth media. On the opposite end, at 1.5 cm and 2.0 cm distances, results were even lower, which could have been caused by more water, sucrose and nutrient deposits that could lead to relief in explants (Figure 3). Yıldız (2000) reported that shoot number per hypocotyl was 2.10 in 'Ariane' and

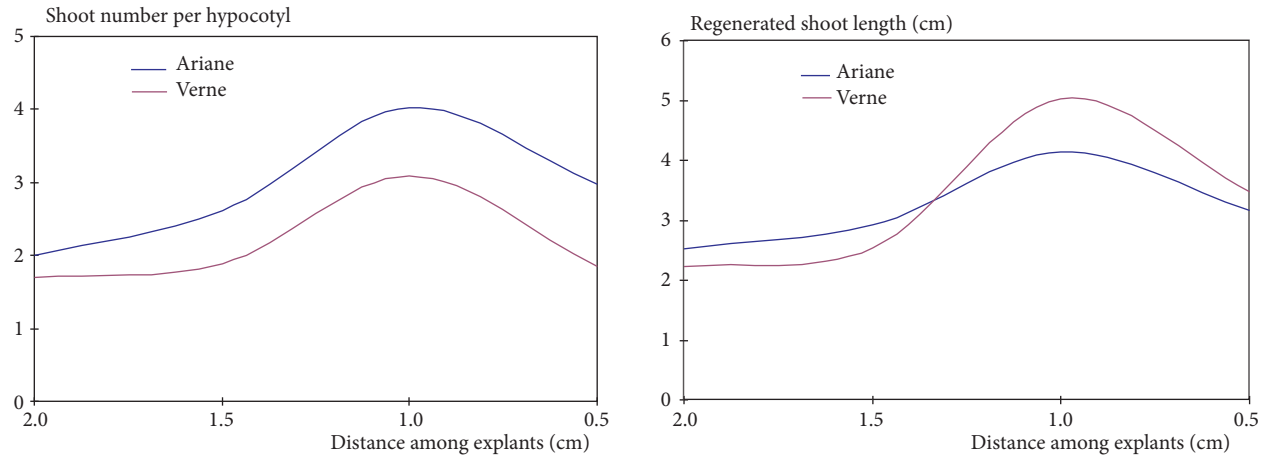

Figure 3. Competition-stress curve of cultivars 'Ariane' and 'Verne' with respect to shoot number per hypocotyl and regenerated shoot length.

2.60 in 'Verne'. Although the same growth regulators composition was used, by utilising the competition among explants, shoot numbers per hypocotyl were found to be higher in both cultivars than in the study conducted by Yıldız (2000).

The highest total shoot numbers per petri dish were again recorded from 1.0 cm culture spacing in both cultivars (Table). Neither shoot regeneration percentage nor shoot number per explant is by itself an indicator of the success of tissue culture studies. Rather, 'total shoot number per petri dish' is the parameter that indicates the success of both shoot regeneration percentage and shoot number per explant for related genotype under in vitro conditions.

Leaf chlorophyll content, which plays a critical role in plant growth and development (Yang et al., 2010), is considered a sign of the photosynthetic capacity of tissues (Emerson, 1929; Pal & Laloraya, 1972; Wright et al., 1994; Nageswara et al., 2001) and its amount can change under stress conditions (Rensburg & Kruger, 1994; Kyparissis et al., 1995; Jagtap et al., 1998). Gireesh (2009) has reported that chlorophyll can be used to measure growth. In total chlorophyll content, the highest values were obtained at 1.5 cm culture spacing in both cultivars, in contrast to shoot number per hypocotyl, regenerated shoot length, and total shoot number per petri dish, which all had their highest values at 1.0 cm spacing. This could be due to the fact that the closely packed shoots at 1.0 cm culture spacing in a petri dish could easily be shaded each other. Our findings were supported by Fleischer (1935) who reported that the intensity of incident light affects the chlorophyll content of the chloroplasts where photosynthesis takes place. The highest total chlorophyll content values were 386.4 μg g^{-1} fresh tissue in 'Ariane' and 396.4 μg g^{-1} fresh tissue in 'Verne' (Table).

Establishment of an efficient regeneration protocol is a prerequisite for the application of biotechnology to crop improvement. Fatima et al. (2009) have reported that growth and morphogenesis are controlled by the types and concentrations of plant growth regulators in plant cell culture. In order to obtain high frequency adventitious shoot regeneration for related genotype, we have tried to determine the correct concentrations and combinations of auxins and cytokinins. To the best of our knowledge, this is first study reporting that adventitious shoot regeneration capacity could be increased not only by determination of correct concentrations and combinations of auxins and cytokinins in growth medium but also by encouraging explants into competition by adjusting culture spacing. Furthermore, the protocol presented in this study could easily be used for other crops in vitro.

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