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VARIABILITY OF ANATOMICAL AND MORPHOLOGICAL TRAITS OF *PINUS NIGRA* AND *PINUS SYLVESTRIS* SEEDLINGS AFFECTED BY DIFFERENT CONTAINER TYPE

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ABSTRACT

In the paper was analysed the influence of three different container types, used for cultivation of *Pinus nigra* and *Pinus sylvestris* seedlings, on dimensions of their anatomical (resin ducts width, resin ducts number, tracheid number, tracheid width, wood rays height) and morphological (height, root collar diameter, sturdiness coefficient) elements, and on proportion of wood, bark and pith, as well. Two-factorial ANOVA showed that container type affects a lot all investigated anatomical traits by both species, but on the other side, these species varied between each other just in terms of tracheid width and wood rays height. Based on descriptive statistics, significantly lower values of all studied anatomical elements were recorded by biodegradable compared to plastic containers. As for *P. nigra* seedlings, they showed the best anatomical performance in Plantagrah I, while Hiko V-120 SS was the most suitable for *P. sylvestris*. The highest proportion of pith and bark was recorded in biodegradable container. As for morphological parameters, such as height and root collar diameter, higher values were recorded by plastic containers.

KEYWORDS: *Pinus sylvetris*, *Pinus nigra*, anatomical and morphological traits, biodegradable container, plastic containers.

INTRODUCTION

In terms of nursery production technology, there are still different opinions related to which seedling type is better and more efficient after outplanting – bareroot or container seedlings. There are a lot of advantages of container seedlings: higher survivar rate during production and later during outplanting, lower physiological shock of seedlings during replanting, extended period of planting, mechanized production, and possibility of degraded sites afforestation (Elam 1981, Matić et al. 1996, Ocvirek 1997, Orlić 2000). As for production of forest species seedlings, smaller containers are much used according to their lower price, easier handling, and higher number of the plants per unit area which can be produced in these container types (Stilinović 1991). Small containers are mainly suitable for seedling producing of species with small seed (Ivetić 2013, 2021), while producing of seedlings with big seed and strong root system demands large containers (Ocvirek 1997, Topić et al. 2006, Popović et al. 2014). The production of container seedlings has increased considerably in the last decade (Dominguez-Lerena et al. 2006). Seedling development directly depends on the growing regime in a nursery since the morpho-anatomical properties of the root system are directly related to the availability of both water and nutrients (McConnughay and Bazzar 1991). Consequently, characteristics of container, such as volume, diameter at the top of container, depth and growing density influence the physiology and morphology of seedlings both in the nursery and in the field after outplanting (Marien and Drovin 1978). Although differences in species response exist (McConnughay and Bazzar 1991), generally, larger container volumes have greater water and nutrients availability, along with more space for root development. Consequently, larger containers generally enable better seedling growth (McConnughay and Bazzar 1991, Hsu et al. 1996) and survival (Matthes-Sears and Larson 1999) postplanting. The growing density also influences seedling development. High density leads to plants with small stem diameter and less height growth following outplanting (Marien and Drovin 1978, Landis et al. 2010). Seedlings producing in containers have also some disadvantages such as root deformation which is caused by the limited rooting volume (Stilinović 1991, Ivetić 2013). These deformations can affect seedling performance several years after outplanting (Marien and Drovin 1978, Halter and Chanway 1993, Lindstrom 1990), although the degree of deformation varies within and between species (Kinghorn 1978).

Among the morphological characteristics of the seedlings, root collar diameter is considered the most important (Grossnickle 2012, Popović et al. 2014). Larger root collar diameter is generally associated with larger seedlings that are better adapted to dry sites than seedlings of smaller diameter (Andivia et al. 2021); improved growth leads to more resource mobilization (Villar-Salvador et al. 2012) that supports drought stress avoidance (Grossnickle 2005). In container nurseries, larger root collar diameter is generally achieved by growing seedlings in larger containers that can provide more resources to individual plants along with lower production densities that favour radial growth (Landis et al. 2010). Pinto et al. (2016) showed that *Pinus ponderosa* seedlings with longer taproots produced in larger containers did not have any advantage on sites with balanced water content during growing season, but they were better adapted to dry, rocky sites. From hystological point of view, primary anatomical structure of the root and trunk are pretty similar – it is characterized by vascular tissue organization where xylem and floem bundles are changing and they are all situated in the pith region at the beggining (Blaženčić 1979).

The scope of the paper was to investigate the influence of container type, two commonly used for forest seedlings production and one new container type, on anatomical and morphological variability of Scots and black pine seedlings and to study how this affects cambial activity and xylogenesis onset by researched species.

MATERIAL AND METHODS

There were used three container types: paper (for single use), Plantagrah I (Bosnaplast 12) and Hiko V-120 SS (Tab. 1). The containers are filled with a mixture of Pindstrup (Pindstrup Mosebrug A/S, Denmark) organic substrate and perlite in a volume ratio of 2:1. Containers were filled manually during sowing at the beginning of the growing season 2021. Provenances of *P. nigra* and *P. sylvestris* seeds originate from western Serbia (Zlatibor mountain).

Tab. 1: Characteristics of containers used for sowing black and white pine seeds.

Container characteristics	**Container type**		
	Paper	**Plantagrah 1**	**Hiko V-120 SS**
Cell shape	circular	hexagonal	square
Cell volume (cm^3)	151	120	120
Depth (mm)	120	120	110
Plant density (n/m^2)	400	660	526
Material	baking soda paper	hard plastic	hard plastic
Drain hole	bottom-open	at the bottom	on the bottom and on the side walls
Cell wall	smooth	smooth	smooth
Dimensions of the cassette (mm)	40 x 120	360 x 250 x 120	352 x 216 x 110
The number of cells in the cassette	20	55	40

Watering was regularly conducted, while foliar fertilization started in the middle of the growing season until its end. Seedlings were also treated with the fungicide Previcur in order to protect from pests attack. It should be noted that Previcur is a systemic fungicide that provides proven control of damaging deseases in young plants, before all. It quickly penetrates the leaf and stem surface and moves throughout the plant to protect new growth. At the end of the growing season sample of 50 seedlings per species and per container type were measured in order to determine height and root collar diameter. As for the morphological analyses, for each species, per container type, 50 seedlings were measured. The height was measured with a ruler with an accuracy of 0.1 cm and the distance from the root collar to the terminal bud was determined. Root collar diameter was measured at the level of transition from the root to the aerial part, using a digital calliper, with an accuracy of 0.1 mm. From the measured heights and root collar diameters of the seedlings, the Roler's sturdiness coefficient was determined as relation between these two parameters (h/D, Ivetić 2013).

At the same time, sample of 5 seedlings per species and per container type were lifted from containers and prepared for laboratory analysis. As for anatomical analyses (Fig. 1), they were carried out on thin cross-sections that are obtained by previously softening the complete material which was firstly kept in boiling water and then in the mixture of water, glycerol and ethanol in the same proportions. The samples were then cut using sliding microtome in transversal sections 20-25 μm thick. Resin ducts diameter (RDD) was measured with 50 replicates for both species and all container types. This parameter was calculated in two perpendicular directions and based on this was obtained the mean value. Number of resin ducts (NRD) and number of tracheids (NT) were measured with 5 replicates. When it comes to methodology of determining the number of tracheids, five visible areas of 1 mm^2 were selected at the beginning, and then the number of tracheids in each visible area was counted. The number of resin ducts was measured so that all those located within one cross-section were taken into account. Tracheids width (TW) and wood rays height (WRH) were measured with 150 replicates. Wood rays height was calculated from the distance between the pith and the bark, while width of initial tracheids was measured both longitudinally and transversely and based on this was obtained the mean value. The proportion of some segments such as wood, bark and pith was calculated in relation to the root collar diameter. All of these parameters (wood %, bark %, pith %) were calculated with 10 replicates.

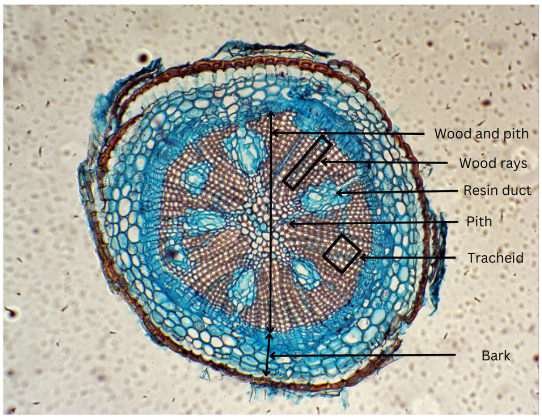

Fig. 1: Measured anatomical traits in container seedlings of P. nigra and P. sylvestris.

Statistical analyses

The data was analysed using two-factorial analysis of variance (ANOVA), and the significance of differences between tree species and type of the containers used were tested by Tukey's HSD (honestly significant difference) test. Significance was determined at $p < 0.05$ throughout. Normality of dependant variables distributions were assessed by Shapiro-Wilk's test. Statistical analyses were performed using statistical software package STATISTICA 12 (StatSoft, Inc. 2012). It should be noted that parameters describing the number of resin ducts and the number of tracheids were transformed by square-root transformation to achieve normality in frequency distribution (Kovačević et al. 2021).

RESULTS AND DISCUSSION

Two-factorial ANOVA showed that type of the container had dominant and highly significant effect both on *P. nigra* and *P. sylvestris* anatomical traits (Tab. 2). In contrast, studied tree species differ significantly only in terms of tracheid width (TW) and wood rays height (WRH). Similar results have been observed for the proportion of wood, bark and pith, which is found to be the most notably influenced ($p < 0.001$) by the container used. Significant effect of "tree species by container type" interaction was observed for the following anatomical traits: resin ducts diameter (RDD), tracheid width (TW) and wood rays height (WRH), as well as proportions of wood and bark. As for morphological parameters, type of the container notably affected both height (H) and root collar diameter (RCD), while sturdiness coefficient (SQ) showed to be statistically insignificant. Among morphological elements, significant effect of "tree species by container type" interaction was observed just for the height.

Tab. 2: Results of analysis of variance showing the effect of tree species (T), type of the container (C), and their interaction (T × C) on the studied anatomical and morphological traits, and the proportion of wood, bark and pith.

Effects	Tree species (T)	Container type (C)	Interaction T × C
RDD	0.11^{ns}	53.98***	5.27**
NRD	0.54^{ns}	40.48***	0.39^{ns}
NT	0.21^{ns}	24.78***	1.33^{ns}
TW	250.34***	407.27***	201.80***
WRH	83.50***	533.24***	53.86***
Wood	2.88^{ns}	45.80***	6.20**
Bark	0.26^{ns}	57.86***	10.71***
Pith	6.94*	9.82***	0.56^{ns}
H	123.29^{***}	10.78^{***}	6.09^{**}
RCD	11.77^{***}	5.74^{**}	1.97^{ns}
SQ	23.34^{***}	0.07^{ns}	1.14^{ns}

Legend: (ns) non-significant; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Based on obtained results (Tab. 3) we can deduce that values of all analyzed anatomical elements are much smaller in paper compared to Plantagrah I and Hiko V-120 SS containers. As for *P. nigra* seedlings produced in Plantagrah I container, they had the greatest mean values of tracheid number, tracheid width and wood rays height, while on the other side the greatest resin duct diameter and number was recorded by Hiko V-120 SS container. By *P. sylvestris* seedlings, values of all anatomical parameters except tracheid width were largest by Hiko V-120 SS container.

If we compare values of anatomical traits for both species for Hiko V-120 SS container, there is significant difference just for tracheid width. The results showed that seedlings of *P. nigra* and *P. sylvestris*, cultivated in Plantagrah I, differ significantly in terms of tracheid number, tracheid width, and wood rays height – interestingly that values of all mentioned elements are greater by *P. nigra*. Unlike Plantagrah I and Hiko V-120 SS containers whose values of almost all anatomical traits are greater by *P. nigra*, seedlings produced in paper containers show completely different pattern. Apart from wood rays' height, all other elements by individuals from paper container have greater values by *P. sylvestris*. Seedlings of both species produced by Hiko V-120 SS, Plantagrah I have significantly higher number of resin ducts per unit area, and much wider resin ducts lumen compared to paper container. As a result, a multi-layered and powerful anatomical and physical barrier, formed by Plantagrah I and Hiko

V-120 SS containers protects seedlings from pests and disease attacks, so these plants are more resistant to environmental stress.

As for tracheid, the same pattern was recorded – number and width of tracheid was much bigger by plants originate from these two containers which means that water conduction efficiency by these individuals is also much higher, so they will potentially be better adapted to dry site conditions. Wood rays are also much higher by plants cultivated in aforementioned two containers, so they have significantly higher capacity of storage and accumulation of nutrients compared to plants from paper containers.

Tab. 3: Mean values and standard deviations of anatomical and morphological traits investigated in P. sylvestris and P. nigra seedlings cultivated in three types of the containers. Differences between values of the same trait that are labelled with the same letter are not statistically significant at $p < 0.05$.

Type of the container	Hiko V-120 SS		Plantagrah I		Paper	
	P. sylvestris	***P. nigra***	***P. sylvestris***	***P. nigra***	***P. sylvestris***	***P. nigra***
RDD (µm)	15.20±6.08 ab	16.82±4.60 a	14.24±2.34 b	14.86±3.31 ab	11.47±1.96 c	9.68±3.06 c
NRD	13.2±2.05 a	13.2±3.90 a	10.4±0.55 a	10.4±0.61 a	6.8±0.45 b	5.8±1.10 b
NT	683.6±181.8 a	637.5±109.7 a	634.0±93.2 a	785.0±157.7 a	362.0±109.2 b	340.0±114.0 b
TW (µm)	1.96±0.38 d	2.39±0.55 b	2.19±0.45 c	3.49±0.67 a	1.87±0.42 d	1.63±0.23 e
WRH (µm)	40.4±10.80 b	38.6±9.12 bc	36.7±8.60 c	48.4±8.03 a	20.4±5.07 e	25.1±4.22 d
H (cm)	7.87±1.01 c	10.43±1.25 a	8.08±1.78 bc	11.06±1.61 a	7.77±1.78 c	9.06±1.23 b
RCD (mm)	1.28±0.19 b	1.49±0.24 a	1.37±0.22 ab	1.51±0.21 a	1.27±0.31 b	1.30±0.27 b
SQ	6.25±1.12 bc	7.14±1.05 ab	5.97±0.84 c	7.40±1.00 a	6.43±2.50 abc	7.11±1.22 ab

Legend: RDD – resin ducts diameter; NRD – number of resin ducts; NT – number of tracheid; TW – tracheid width; WRH – wood rays height; H – height; RCD – root collar diameter; SQ – sturdiness coefficient.

The analyses of morphological elements also showed lower values for height and root collar diameter by paper compared to the other two containers (Plantagrah I and Hiko V-120 SS) for both investigated species. On the other side, SQ has the highest value in paper container for *P. sylvestris*, but generally SQ is satisfactory for each seedling species and container type. The seedlings of *P. nigra* cultivated in Plantagrah I containers showed the highest values of all studied morphological traits. At the species level, both *P. nigra* and *P. sylvestris* showed the best height and root collar diameter in Plantagrah I containers (Tab. 3).

Proportions of wood and bark were insignificantly different in two species, whereas the proportion of the pith were found to be notably higher in *P. sylvestris* seedlings (Fig. 2). The proportion of the wood ranged between 53.9-65.6% and 57.6-65.3% in *P. sylvestris* and *P. nigra* seedlings, respectively, and was the lowest in paper containers. The largest proportion of the wood was recorded in containers Hiko V-120 SS for *P. sylvestris*, and Plantagrah I for *P. nigra*. In contrast, the seedlings cultivated in paper containers were characterized by the largest proportions of bark and pith. Moreover, the smallest proportion of pith was recorded in seedlings of both species grown in Plantagrah I containers. Seedlings of both species cultivated in Plantagrah I and Hiko V-120 SS containers are characterized by higher wood proportion which is related to their earlier xylogenesis onset and most intensive cambial growth compared to plants produced in biodegradable containers.

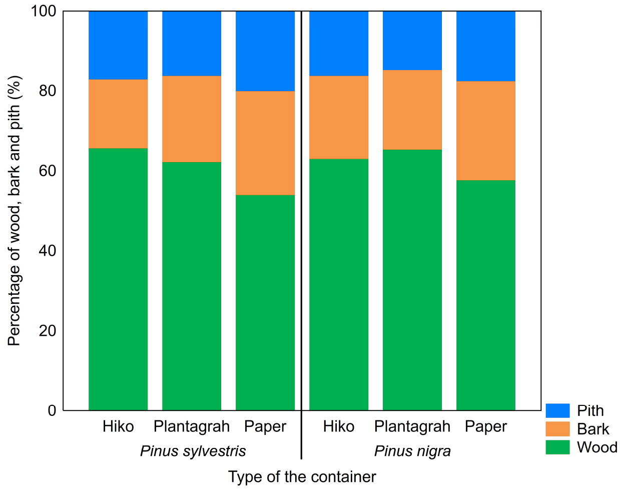

Fig. 2: Proportion of wood, bark and pith in P. sylvestris and P. nigra seedlings cultivated in three types of the containers.

Montagnoli et al. (2021) found increases in the amounts of vascular cambium cells depending on container size. This may affect an earlier onset of vascular cambium activity in larger containers or may be associated with an overall higher cambial activity (Montagnoli et al. 2019). Regardless, the thickness of the xylem area was, however, similar among the two container sizes and rays followed the same patern (Montagnoli et al. 2021). In our paper, container type has a big influence both on wood proportion and wood rays height – these parametres have much higher values in Plantagrah I and Hiko V-120 SS compared to paper containers independing on species. Interestingly that an additional cambial activity by seedlings originating from larger containers contributed to double number of resin ducts forming compared to seedlings from smaller containers (Montagnoli et al. 2021). Our results showed that number of resin ducts is closely dependant on container type – this value is two times greater by Plantagrah I and Hiko V-120 SS compared to paper containers. Exogenous and indigenous factors readily influence resin ducts formation by the cambium (Werker and Fahn 1969). Resin ducts occur as a response to potential damage and are mostly traumatic created in case of mechanical injury (Vazquez-Gonzales et al. 2020). A greater number of resin ducts in tree species, including pines (Kane and Kolb 2010) affects a higher resin flow and a wider anatomical barrier for insects attacks and diseases (Rodriguez-Garcia et al. 2014, Hood and Sala 2015). O'Neill et al. (2002) found positive correlation between resin duct features and aridity. There are some factors such as limited carbon accumulation caused by environmental stress that affect smaller radial dimensions and abundance of resin ducts that influences to plant defense mechanism (Vazquez-Gonzales et al. 2020). Montagnoli et al. (2021) examined that more vascular cambium was produced in larger versus smaller containers and despite xylem thickness was unaffected, number of resin ducts increased by larger containers which is related to high plasticity in cell development by vascular cambium. Our results confirmed this hypothesis because we found greater size and abundance of resin ducts by Plantagrah I and Hiko V-120 SS containers whose plant density is greater compared to paper containers.

In our paper, from hystological point of view (Fig. 1), the organization of anatomical elements is very similar independing on container type and tree species. As for the resin ducts, they are ellipsoidal or irregularly circular in shape, and in terms of arrangement, they are scattered within approximately regular concentric series. Wood rays are radially distributed from pith to bark and consisted of cells which are, less or more, with the same lumen diameter and cell wall thickness along the entire length. The secondary meristem in the form of cambium is multi-layered and built from several groups of initial cells capable of differentation and division. Tracheids elongated and narrow with pointed tips. The only hystological difference that can be observed refers to the number of vascular cambium cells, which is lower in paper compared to the other two container types. This occurs as a consequence of different density per area by plants cultivated in these container types. Due to the aforementioned differences, cambial activity and the process of xylogenesis (wood formation) starts earlier and takes place more intensively by Plantagrah I and Hiko V-120 SS containers which is in direct relation to the number of vascular cambial cells. As a consequence, the number of resin ducts which is directly correlated with cambial activity is almost double greater by aforementioned containers which means that physical barrier for pests attacks and diseases is more powerful by seedlings produced in this container type.

Jelić et al. (2014) investigated the influence of the container size on the dimensions of the morphological traits of one-year old seedlings of *Pinus pinea* and found that a larger container volumeaffects the increase of the values of height and root collar diameter of the seedlings. The same authors believe that seedlings of the mentioned species produced in larger containers can be used for afforestation of more difficult sites, because the quality of the seedlings largely depends on the dimensions of the morphological traits, and primarily on the root collar diameter. However, they note that it is very important to monitor the development of the root system and its potential deformations. Dominguez-Lerena et al. (2006) also studied the influence of container properties on the development of *Pinus pinea* seedlings both in the nursery and at the place of permanent afforestation, and found that the volume of the container is positively correlated with the seedlings height, and especially with the root collar diameter, which is the main indicator of the seedlings quality. Mijatović et al. (2022) monitored the influence of the container size on the morphological features of one-year old pedunculate oak seedlings and came to the same conclusions – the larger container volume and thus the lower plant density per unit area affects higher values of height and root collar diameter of the seedlings. The results of all the abovementioned studies on the influence of the container volume on the behaviour of the morphological characteristics of the seedlings, both conifers (*Pinus pinea*) and hardwoods (*Quercus robur*) do not coincide with results obtained in our paper – namely, we found that paper container, which has a slightly larger volume than the other two containers, but also a significantly lower plant density per unit area, is characterized by lower values of height and root collar diameter in both investigated species. Influence of container type on *P. nigra* seedlings and their field performance is well documented by Devetaković et al. (2017), so one year old seedlings can be better than two years old seedlings if container type and nursery treatment are more suitable.

Bobinac and Vilotić (1998) studied multiphase growth of Turkey oak seedlings and its correlation to morpho-anatomical wood properties. Based on their results, it was deduced that values of all analyzed anatomical parameters apart from bark thickness (primary xylem width, pith width, vessels diameter, vessels abundance) increase with cambial age. In our paper, the largest bark proportion was found by paper container independing on wood species which means that lower plant density and greater space for their development positively affects bark content. Bobinac and Vilotić (1995) also analysed how multiphase growth of *Fagus sylvatica* offspring affects its morpho-anatomical properties and found the increasing of each anatomical parameter during next growing season.

Montagnoli et al. (2021) observed anatomical structure of seedlings of *Pinus ponderosa* root and established that, from hystological point of view, there was no influence of container size neither on primary (meristematic zone) nor on secondary (middle zone) root zone. However, in collar root zone were found some anatomical differences between seedlings grown in smaller and larger containers. There was namely concluded that seedlings in large containers had more than twice the number of undifferentiated vascular cambium cells than did seedlings in small containers, and also the number and position of resin ducts was affected by container size.

Martin et al. (2010) studied the influence of different provenances on wood anatomical traits of *Pinus sylvestris* and deduced that ecological conditions play a very significant role in dimensions of anatomical elements. These authors namely found that trees grew in the driest region were characterized by large tracheid lumens suggesting more efficient water conduction. They also had thick cell walls which would reduce the risk of cavitation, as well as a high ray tracheid frequency, implying greater water storage capacity in the sapwood. At higher altitudes, tracheid width and resin duct diameter tended to be smaller.

On the other hand, Esteban et al. (2012) noted that *Pinus nigra* trees in arid environment were characterized by shorter tracheids which is affected by their poor growth, while severe winter cold spells were strongly associated with larger radial resin ducts, creating a powerful, preformed defence system.

CONCLUSIONS

There was investigated variability of anatomical (number and diameter of resin ducts, number and width of tracheid, wood rays height) and morphological elements (height and root collar diameter), and proportion of wood, bark and pith, as well, by one-year-old *P. sylvestris* and *P. nigra* seedlings cultivated in different containers (biodegradable and two plastic). Based on obtained results, we can deduce that dimensions of all anatomical and morphological traits are much smaller in paper container compared to Plantagrah I and Hiko V-120 SS containers. From anatomical point of view, the most suitable container was Hiko V-120 SS for *P. sylvestris*, and Plantagrah I for *P. nigra*, respectively. On the other side, both investigated species showed the best morphological performance in Plantagrah I. The highest bark and pith proportion was recorded in paper container independing on species unlike wood proportion. Obtained results showed that Plantagrah I and Hiko V-120 SS containers had greater values both of anatomical and morphological traits, which may give them some advantage for afforestation of some harsh sites. This fact, however, has to be considered because there are two significant factors that should be taken into account during species selecting for afforestation purpose – rate of root system deformation and transplant shock that the plant suffers. Therefore we need to have feedback related to outplanting performance in the field and based on survival and vitality rate of the seedlings, we may suggest which container type would be the most suitable for nursery production.

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