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Original Article

Adventitious root formation in branch cuttings of *Taxus wallichiana* Zucc.(Himalayan yew): A clonal approach to conserve the scarce resource

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ABSTRACT

Himalayan yew (*Taxus wallichiana* Zucc.), is an economically valuable plant and critically endangered due to overexploitation for the isolation of Taxol, an exciting anticancer drug from its bark and leaves. Since the species is unisexual and due to its long seed dormancy period and rapid loss of viability coupled with low survival percentage, its natural regeneration from seeds is very poor. As the seed-raised plants add little growth, propagation by stem cuttings was tried under natural conditions and a considerable success was achieved after making use of different auxins (indole-3- acetic acid, indole butyric acid and naphthalene acetic acid) in different concentrations. A randomized block design was adopted for laying the experiment of the present study. Of the 10 treatments studied, IBA at 500 ppm performed best of all the treatments and registered higher callusing percentage, rooting percentage, number of roots, and length of roots in the juvenile shoot cuttings of the species. The results achieved through the application of plant growth regulators by way of adventitious root formation could be useful for the management of this understory coniferous tree species, whether for conservation, habitat restoration, or for the production of Taxol, a promising anticancer agent. The technique evolved will be the most handy, quickest, inexpensive, and can be applied anywhere in its natural habitat for the restoration and restocking of this valuable plant, which is otherwise facing the peril of extinction throughout the range of its distribution including Indian Himalayas.

KEY WORDS: Adventitious root formations, conservation, indole butyric acid, medicinal plant, plant growth regulators, propagation, *Taxus wallichiana*

INTRODUCTION

Conservation of endangered species and their habitats is a priority feature of environmental policies in many countries and international organizations (Oyonarte *et al*., 2008). Humans have inflicted so much ecological damage on the planet that a real concern is how to protect what remains (Larsen and Olsen, 2007). Clonal propagation of branch cuttings provides the advantage of greater genetic uniformity and availability of superior stock in a short period for afforestation works. It also permits the multiplication of a desired tree having superior characters (otherwise called as candidate plus tree). The resulting populations of plants have the same genotype as the original source (ortet) and are called the clone (ramet) (Saini, 1998). It is considered as the only preferred practicable option for the cultivation of a species on large scale with better quality and higher yield, thereby augmenting forest productivity (Aslam and Rather, 2008). Adventitious root formation (ARF) has been successfully used in a program of conservation of genetic resources of some medicinal woody plants in Nigeria (Oni, 1993). The vibrant but fragile forest ecosystems of the Kashmir Himalaya also warrants the development of economical and time-saving means for the proliferation of superior clonal stock (Aslam, 2007). An efficient rooting treatment can lead to a high percentage of rooting and a higher quality of root system (De Klerk *et al*., 1997). Quality involves root number and length, both of which influence the performances of the plants after transfer to field

(Mohammed and Vidaver, 1990). Many factors, during the rooting phase, can cause poor quality of the shoots at the time of planting (Hartmann *et al.*, 2002). To cater to such needs the plant growth regulators (PGRs) have long been recognized as great fillip (Nanda, 1970; Blazich, 1988; Davis and Haissig, 1990; Nanda and Kochhar, 1995).

The *Taxus wallichiana* Zuccarini (syn. *Taxus baccata* subspecies *wallichiana* (Zucc. Piger), commonly known as Himalayan yew belongs to family Taxaceae, is an understory temperate gymnosperm of high medicinal value and ethnobotanical importance. Although the members of this family are apparently similar to the conifers, due to the absence of cones and resin ducts, are placed in a separate order named as Taxales. It has been in frontline focus, as a terrible fate befell on this conifer more recently because of its overexploitation and excessive destructive harvesting from its natural stands (Aslam *et al.*, 2009). Consequently, the species has to bear the brunt of excessive clipping of foliage and bark removal, as a prime source of various taxoids presented as a highly effective anticancerious drugs (CITES, 2009). It is originally, the Pacific yew (*Taxus brevifolia*), which has entered the halls of fame, because research into its chemistry resulted in the discovery of a well-known anticancer drug, Taxol (Stephen, 1991). Subsequently, the taxol and related bioactive taxoids have been reported from the various species of the genus, including *T. wallichiana* (Bala *et al.*, 1999; Prasain *et al.*, 2001; and Wani *et al.*, 1971). As such, the large-scale foliage and bark collection would definitely do more harm than good and could hence erode the very genetic resource base of the species. Since the occurrence of the species has reduced to a great extent and is on its fade out; a conservative approach is required to protect this scarce resource globally.

Taxus is genus of 10 species and taxonomically regarded as the troublesome genus because of the variability and overlapping nature of the limited number of morphological features that are used to distinguish the species (Edward, 1998). Classically, the 10 renowned species of genus *Taxus* are *T. baccata* (European yew or English yew), *T. brevifolia* (Northwest Pacific yew), *Taxus canadensis* (Canadian yew), *Taxus chinensis* (Chinese yew), *Taxus cuspidata* (Japanese yew), *Taxus floridana* (Florida yew), *Taxus sumatrana* (Sumatran yew), *Taxus globosa* (Mexican yew), *Taxus celebica* (Celebes yew), and *T. wallichiana* (Himalayan yew) (Paul *et al.*, 2013). The range of *T. wallichiana* comprises Afghanistan, Bhutan, China, India, Pakistan, Indonesia, Malaysia, Myanmar, Nepal, Vietnam, and the Philippines (Riedl, 1965). In the Indian Himalayas, the species occurs in the Northern-Western States of Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Assam, and Arunachal Pradesh at altitudes between 1800 and 3300 m (amsl) and in the hills of Meghalaya and Manipur at an altitude of 1500 m (amsl) (Karki and Gupta, 2000; Sahni, 1990). In Kashmir Himalaya, the species shows its prevalence more in Gulmarg, Baramulla, and Pahalgam regions and also grows extensively in the inner areas of Vaastoorwon forests at Tral, of Awantipora Forest Division (Aslam, 2014). Likewise, the species also grows in Sudhmahandev, Patnitop, Sannasar, Sarthal, and Dudu Basantgarh areas of Jammu Province (Sharma, 2014). The Himalayan yew's habitat is mostly characterized by mixed coniferous forest species (*Abies pindrow*, *Abies spectabilis*, *Cedrus deodara*, *Pinus wallichiana*, and *Picea smithiana*) or broadleaf tree species (*Prunus padus*, *Quercus semicarpifolia*, *Aesculus indica*, *Betula utilis, Acer caesium*, *Rhododendron arboretum*, and *Juglans regia*) (Aslam *et al.*, 2009) and grows as scattered individuals and small groups of trees on or near streams banks and river margins, canyon bottoms, and wet-shaded ravines and in other moist habitats (Crawford and Johnson,1985).

The extracts of *T. wallichiana* are known to be a source of a drug, called *Zarnab*, which is very frequently prescribed in the Unani System of Indian Medicine as sedative, and for the treatment of bronchitis, asthma, snake bites, epilepsy, scorpion stings, diarrhoea, severe biliousness, giddiness, feeble, and falling pulse, besides application as an aphrodisiac (Anonymous, 1976; Mulliken and Crofton, 2008). The leaves of this plant are also credited with antispasmodic properties and are employed for the treatment of hysteria, nervousness, carminative, and antimalaria properties (Chauhan, 1999). Before independence in Jammu and Kashmir, especially in Ladakh, *Taxus* bark was used as a substitute for preparing herbal teas locally known as "Namkeen Chai" and frequently used for curing coldness of extremities (Aslam, 2006). The pulp of fleshy bright-scarlet aril is gelatinous and very sweet tasting and is used in jams, ice creams, as long as the toxic seed is removed and can also be added in cosmetics, such as hair lotions, rinses, beauty and shaving creams, and dentifrices (Aslam *et al.*, 2009; Edward, 1998). The arils, removed from the seeds, have diuretic and laxative effects (Orwa *et al.*, 2009). The wood of *Taxus* is considered as the hardest of all coniferous woods and is valued for its strength, durability, and decorative character (Sabina, 2006). It is also highly resistant to rot and insect attack and can be worked, polished, and stained excellently (Dar and Dar, 2006). The colorful wood (red heartwood and white sapwood), is used for cabinet work, candle sticks, and other fancy articles, such as handles of knives, doors, and back of combs. It is used for furniture, veneers, parquet flooring, and paneling and for gates, fences, pegs, and various art objects (Oldfield *et al*., 1998). In J&K, the fine-grained elastic wood was formerly valued for making archery bows and fine musical instruments (Aslam *et al*., 2004; Vance and Rudolf, 1974). It is also used for making ploughs, carts, axles, spindles, and also for the construction of beehives (Purohit *et al*., 2001). It is used as a cheaper type of gun and rifle wood (Troup, 1921). The wood is also used in the cremation of dead bodies and its bark also finds use in making essence sticks (Aslam, 2006; Gamble, 1922). As the poisonous contents are usually low in the tree, its foliage is also used under extreme conditions (particularly in winter and early spring) as cattle fodder, when the snow reduce access to grazing (Aslam, 2016). Ironically, it is not for the timber but for the leaves and bark for which the anticancer yew trees are in the threat of extinction.

The importance of the tree has further increased in the recent times with the yield of Taxol, which is extracted from its bark and leaves, and demonstrated broad antitumor activity against the breast, liver, lung, blood, prostrate, head and neck, gynecological cancers, and Kaposi's sarcoma - an AIDS-related cancer (Cragg *et al*., 1993; Donehower and Rowinsky, 1993). In addition, taxol may prove useful against other non-cancer disorders including polycystic kidneys diseases (Woo *et al*., 1994). It was first commercially developed by the pharmaceutical company Bristol-Myers Squibb Company-Princeton, NJ with the generic name paclitaxel and sold under the trademark Taxol (McCoy, 2004; Wheeler and Hehnen, 1993). Taxotere is a registered trademark of Rhone-Poulenc Sante-Paris, France. Its generic name is Docetaxel. At best, 1 kg of dried yew bark yields only 50-150 mg of pure Taxol and for producing a gram of the substance three trees each at least 60 years are required and 1 mg of it costs $60 in the world market (Stephen, 1991), such a high monetary incentives have lead to reckless exploitation and an immediate threat to the prosperity of this precious plant. Even though severe restrictions have been imposed by the State Forest Departments on the removal of *Taxus* trees from the forest areas, illegal debarking, removal of shoots, and even tree continues unabated. This clandestine trade has wiped out yew trees from the whole forests of Jammu and Kashmir State and elsewhere (Aslam *et al*., 2004). Owing to these reasons, *T. wallichiana* was listed in Appendix 2 CITES (Thomas and Farjon, 2011) in 1995. It was assessed as data deficient (DD) by IUCN in 2006 (Paul *et al*., 2013).

The conservation situation of *T. wallichiana* is becoming worsening due to the destruction of its natural habitat and collection of its leaves and bark for medicines since 1970s, the distribution of this species have been progressively reduced (Fu, 1991). Conservation of this endangered species and its habitats are becoming urgent and basic studies are needed for it. Any contribution to restore the population of threatened species is a milestone to conserve valuable species for the greater benefits of humankind. Although the regeneration of *Taxus* through seeds is possible (Pandey *et al*., 2002), but faces constraints mainly because of slow process, rapid loss of seed viability coupled with low survival percentage (Rajewski *et al*., 2000). So as to counter its continuous degradation and to augment its natural regeneration and large scale cultivation, attempts for its artificial regeneration are urgently called for. Since *T. wallichiana* is shy to root (Aslam, 2006), vegetative propagation through cuttings, was attempted by the State Forest Research Institute-J&K, (India) after treated with PGRs of different concentrations in the natural conditions within its original climatic zone. The use of PGRs to induce adventitious rooting in branch cuttings of most forest tree species is well known (Husen and Pal, 2006), and could be a feasible way to achieve conservation and restoration goals as well as to meet the demand for paclitaxel (Heinstein and Chang, 1994), for which the species is facing the peril of extinction (Aslam, 2016).

However, a well-documented scientific study in this species with regard to efficient hormonal treatment and exact concentration is fragmentary/missing. Keeping in view the small population size, poor regeneration, slow propagation, habitat specificity, destructive harvesting, overgrazing, high value of utilization, and habitat loss, the present study was planned to raise adequate supply of clonal stock through adventitious rooting with desirable traits, so as to achieve suitable propagation protocol for this under pressed species, for use in field for the propagators of Jammu and Kashmir State and elsewhere.

MATERIALS AND METHODS

Experimental Site

The experiment has been carried out at the Forest Research Center, Seer of State Forest Research Institute (SFRI), Jammu and Kashmir, India, which is situated about 2.5 km from Seer village on Khanabal-Pahalgam-Shri Amarnathji cave road and 12 km from main Anantnag town. The nursery is located in compartment no.03/Lidder Forest Division at an elevation of 1,900 m above mean sea level (amsl) on the Western aspect and lies at 34° -17' N latitude 75° -20' E longitude, receiving mean rainfall of 2012.7 mm annually. Severe frost and snowfall is a usual feature in this area from December to February and hence experiences a wide range of monthly temperature variations ranging from - 5.0°C (January) to 35.3°C (June).

Collection and Preparation of Cuttings

The trees of *T. wallichiana* growing in the compartment-37/L in Pahalgam range of Lidder Forest Division were sheared by removing their juvenile apical shoots and brought to the experimental site in clean and moistened gunny bags to avoid them from desiccation (3rd week of March, 2008). Subsequently, the cuttings of uniform size (22 cm length × 0.5-1.0 cm diameter) were then prepared from these excised shoots. Needles from the 5 to 8 cm basal portion of these cuttings were removed carefully, while sufficient number of leaves at the upper portion was kept intact.

Treatments of Cuttings

The basis of each cuttings was given slanting cut and dipped in 0.1% aqueous solution of Bavistan containing 50% carbandizam w/w (fungicide-BASF India Ltd.) for 3-5 min to protect cuttings from any chances of fungal infection, subsequently washed with distilled water. These cuttings were subjected to 9 treatments, that is, 3 phytohormones, namely, indole-3-acetic acid (IAA), indole butyric acid (IBA), and naphthalene acetic acid (NAA) (Sigma Chemicals Co., St. Louis, USA) each with three concentrations, namely, IAA, (IAA 500 ppm, 1000 ppm, and 2000 ppm), IBA (IBA 500 ppm, 1000 ppm, and 2000 ppm), and NAA (NAA 500 ppm, 1000 ppm, and 2000 ppm) (Table 1). Before planting, the lower portions of these juvenile cuttings were dipped separately in the appropriate hormonal solutions for 24 h. The cuttings treated with distilled water served as control. Three replicates each of thirty cuttings were maintained for each treatment. The selection of rooting hormone, their concentration, and duration of the treatment was based on the rooting response of the cuttings in our earlier experiments with the species. All the chemicals were first dissolved in 10 ml of ethanol and then making the final volume to 1 l by adding double distilled water.

Propagation Medium and Planting of Cuttings

Immediately following the chemical treatments, cuttings were planted to about 5-6 cm depth, maintaining uniformity, in the perforated polybags (size 20×10 cm) filled with forest soil, sand, and humus (2:1:1), firmed to position, and permitted to develop adventitious roots. The polybags were arranged in a randomized block design under the shade of *P. wallichiana* trees in natural conditions (Figure 1a-c) and watered manually with the help of rose cane on alternate days depending on the weather conditions and rooting medium moisture status without disturbing the experiment. The polybags were kept free of weeds manually.

Recording of Data on Adventitious Rooting in Cuttings

The data on various rooting parameters were recorded by observing cuttings from each replicate of each treatment after 5 weeks of treatment. The final observation on callusing, root emergence, number of roots emerged, and length of roots emerged were recorded after 18 weeks, when the experiment was terminated. All the cuttings with even 1 callus were considered as callused and used for calculating callusing percentage. Cuttings with roots ≥1 mm roots were considered to have rooted and used for calculating rooting percent and accordingly root/roots >2 mm were considered for calculating mean root number and mean root length per cutting.

Table 1: Effect of auxins on induction and growth of adventitious roots in shoot cuttings of *Taxus wallichiana* after 18 weeks of treatment and planting. Results are the mean values of three replicates

Treatment (ppm)	Callusing[1] (%)	Rooting[2] (%)	Roots per cutting[3](mean[4]±SE[5])	Root length per cutting[3](mean[4]±SE[5])
T_1 Control (untreated)	4.30	8.00	3.60±0.14	3.20±0.15
T_2 IAA 500	6.67	40.00	10.21±0.38	8.40±0.14
T_3 IAA 1000	6.00	30.00	11.00±0.17	11.83±0.28
T_4 IAA 2000	4.30	15.00	6.00±2.06	7.20±0.29
T_5 IBA 500	11.3	76.66	12.33±0.66	12.50±0.21
T_6 IBA 1000	4.58	30.00	5.65±0.29	9.50±0.69
T_7 IBA 2000	3.55	20.00	3.20±0.23	6.80±0.24
T_8 NAA 500	8.20	70.30	11.20±0.24	11.95±0.28
T_9 NAA 1000	4.23	30.00	4.20±0.12	9.10±0.29
T_{10} NAA 2000	3.30	25.00	6.30±0.14	8.70±0.27
CD at P=0.05%	1.893	27.006	1.029	1.155

Mean values are significantly different at P=0.05%. [1]Cuttings with even 1 callus were considered as callused, [2]Cuttings with roots ≥1mm roots were considered to have rooted, [3]Root/roots >2 mm were considered for calculating mean root number and mean root length per cutting, [4]Mean: Mean values of the three replicates in each treatment, [5]SE: Standard error of the mean

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and F-tests for significance as per the procedure devised by Panse and Sukhatme, 1967. Critical difference values were calculated for comparing the treatment means at $P = 0.05$.

RESULTS AND DISCUSSION

The data with regard to effect of auxins on induction and growth of adventitious roots in shoot cuttings of *T. wallichiana* after 18 weeks of treatment and planting are depicted in Tables 1 and 2. A look on the data recorded in the present study clearly reveals that the auxins, in general, proved effective on root induction and growth of adventitious roots in stem cuttings of *T. wallichiana* within 18 weeks of treatment and planting (Table 1). In general, it has been observed that among all the three auxins (IAA, IBA, and NAA) tried, treatment T_5 (IBA at 500 ppm) was the most effective for root induction. Lower concentrations (500 ppm) of all the three auxins were found to be better than higher concentrations. The lower concentrations, i.e., IBA 500 ppm, IAA 500 ppm, and NAA 500 ppm tried also showed maximum callusing percentage, rooting percentage, roots per cutting, and root length per cutting except in IAA 500 ppm where maximum root length per cutting was observed in IAA 1000 ppm. The highest rooting of 76.66% was recorded under IBA 500 ppm with 12.33 roots per cutting attaining about 12.50 cm root length. This was closely followed by NAA 500 ppm where a rooting of 70.30% was achieved with 11.20 roots per cutting attaining about 11.95 cm root length. However, control cuttings (untreated) were observed to have lowest callusing percentage (4.30%), rooting percentage (8.00%), and accordingly root number and root length per cutting.

Figure 1: Conserving the scarce resource of *Taxus wallichiana* in Kashmir Himalaya (J&K), India. (a) A medium-sized and densely branched tree of yew, (b) preparation of rooting medium and filling of polybags (author in middle of photo), (c) cuttings planted in polybags for the stimulation of rooting, and (d) representative rooting response of yew cuttings after treated with various plant growth regulators after the 18 weeks of treatment and planting

The ANOVA is presented in Table 2, which clearly shows that all the treatments differed significantly with respect to all the rooting parameters, namely, callusing, rooting, roots per cutting, and root length per cutting. It therefore revealed that treatment T_5 (IBA at 500 ppm) is the best and most efficient hormone and concentration in stimulation of callusing, rooting, higher root number, and greater length of primary roots in the juvenile branch cuttings of *T. wallichiana* (Table 1 and Figure 1a-d). The rooting was very slow and not profuse with T_1 (control). Hence, to induce profuse rooting, treatment of cuttings with the auxins in general and IBA at 500 ppm in particular is found to be beneficial.

A varying degree of success on the rooting with auxins in juvenile shoot cuttings of *T. wallichiana* has been reported from other temperate regions of the country as well (Dubey, 1997; Khali and Sharma, 2003; Mishra *et al*., 2000; Mitter and Sharma, 1999; and Nandi *et al*.,1997). Aslam and Rather, 2008 reported that among all the auxins tried, IBA is the most effective hormone for the induction of higher percentage of rooting in the juvenile cuttings of *T. wallichiana*. Similar results have been found using IBA with a number of other yew cultivars (Eccher, 1988; van Hofi, 1978; and von Korya, 1976) and indicated that hormone treatment was important for a successful propagation and conservation program. These results are also in line with Nautiyal *et al*. (1991), who used

Table 2: ANOVA on effect of auxins on induction and growth of adventitious roots in shoot cuttings of *Taxus wallichiana* after 18 weeks of treatment and planting

Source of variation	MSS			F-value
	Treatment	Replication	Error	
Degree of freedom(df)	9	2	18	
Callusing	17.46	1.05	1.22	14.31*
Rooting	652.91	207.53	248.45	2.62*
Roots per cutting	36.2	0.02	0.37	97.83*
Root length per cutting	23.53	0.48	0.46	51.15*

MSS: Mean sum of square, *highly significant at 0.05% level of significance, ANOVA: Analysis of variance auxins in inducing rooting on branch cuttings of teak and concluded that IBA is the best auxin. Some other researchers (Aslam *et al*., 2007; Blazich, 1988; Chandra and Verma, 1989; Gurumurti and Bhandari, 1988; Nautiyal *et al*., 2004; Pal, 1992; and Singh and Chander, 2001) also reported that exogenous application of IBA and other auxins in promotion of ARF in stem cuttings of many plants including *Taxus*. It is a well-established fact that the application of auxins is necessary for the formation of roots in stem cuttings as they have been found to stimulate cambial activity thereby resulting the mobilization of reserve food material to the site of root initiation (Haissig, 1974; Philips, 1971). Applications of auxins enhanced rooting and root quality in many tree species (Hartman and Kester, 1983). The application of IBA may have an indirect influence by enhancing the speed of translocation and movement of sugar to the base of cuttings and consequently stimulate rooting (Haissig, 1974). It has also been suggested that optimum concentration of auxins is favorable, whereas concentration above optimum is toxic to the root regeneration (Avanzato *et al*., 1998; Chauhan and Reddy, 1974). The higher concentration of all the three auxins IAA, IBA, and NAA had been less effective than lower one for various rooting attributes (Tables 1 and 2 Figure 1a) could be due to similar actions in *T. wallichiana* in the present investigation as well.

Growth regulators (auxins) are involved in plant root formation, lateral bud inhibition, and activation of cambial cells. They have been identified as naturally occurring compounds promoting root formation and synthetic auxins also practically stimulating root formation on cuttings. It is well accepted and confirmed many times that auxins naturally or artificially applied are required for the initiation of adventitious roots on stem cuttings. Division of root initial cells is dependent on either applied or endogenous auxins (Gill *et al*., 2006) and leads to synthesis of root primordial (Nanda, 1970). The leaves present in cuttings supply necessary root-promoting substances (Hartmann *et al*., 1981; Nanda and Kochhar, 1995; and Pant and Joshi, 1999) probably by performing photosynthesis during propagation (Mesen *et al*., 1997; Pant and Joshi, 1999), as is the case in the present study, in which a good number of leaves on the upper portion of the cuttings were kept intact. In the cuttings, where there was no root formation or it was delayed, the survival becomes very difficult or the cutting was likely to die (Dugma, 1988; Puri and Thompson, 1989).

CONCLUSION

Since IBA 500 ppm treatment is proven to be the best rooting hormone and concentration for rooting of juvenile shoot cuttings of *T. wallichiana*. Therefore, it is recommended that this threatened, fragile, and overexploited medicinally important plant species be preserved to the greatest extent possible for the future generations with the aid of auxins in general and IBA at 500 ppm in particular. Keeping in view the development of present technique along with the status, importance, and conservation value of *T. wallichiana,* well-rooted plants could be obtained within a short time; the method is also inexpensive and easy to perform under natural conditions. Such knowledge will aid propagators, geneticists, and tree improvement specialists in selecting treatments that reduce propagation cost by maximizing rooting success. This will in turn help to enhance resource availability for meeting the market demand of the species for the extraction of Taxol, for which the species is exploited recklessly. Hopefully, the present studies will also pave the way to enhance the quality and productivity of forest ecosystems of the Kashmir Himalaya and to compensate the damage caused to this exciting tree species which has included it in the 2000 IUCN Red List.

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