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Effect of the plant growth stimulant zeatin on regeneration capacity of some *Physalis* species *in vitro* culture

O.M. Yaroshko, D.B. Rakhmetov, M.V. Kuchuk

The aim of the study was to find an efficient culture medium for regeneration of *Physalis* species *in vitro* to provide their further propagation *ex vitro* and obtain fructiferous plants from the regenerants. *Physalis peruviana* L., *P. ixocarpa* Broth. (cv. Likhtaryk), and *P. pubescens* L. (cv. Zarynka) were taken as plant material for the research. Plant introduction into culture and regenerant production were carried out *in vitro*; the rooting of mature plants and obtaining plants with ripe fruits took place in a greenhouse and in open ground (*ex vitro*). To obtain regenerants, we used Murashige and Skoog (MC_{30}) medium supplemented with the growth stimulant zeatin (Zea) at a concentration of 0.5–3 mg/l. The growth stimulant 6-benzylaminopurine (BAP) was used to elongate the regenerant stalks, and the growth stimulator α-naphthylacetic acid (NAA) was used to initiate root formation. Plant regeneration frequency and the number of regenerants per explant served as indicators of the efficiency of various zeatin concentrations on the physalis regenerative capacity. The most effective media for the shoot regeneration from cotyledonous leaf explants were MC_{30} + 1 mg/l Zea and MC_{30} + 2 mg/l Zea. Regeneration frequency on these media was 46.15 % and 53.84 % for *P. ixocarpa* (cv. Likhtaryk), 38.46 % and 45 % for *P. peruviana*, and 27 % and 34 % for *P. pubescens* (cv. Zarynka) respectively. The emerged regenerants were separated from explants and transferred to MC_{30} medium supplemented with 1 mg/l of BAP + 0.1 mg/l of NAA for stalk growth and rooting. After a month of cultivation, juvenile plants were obtained. They were transferred to a greenhouse for adaptation, and later to open ground at the experimental plot. Three months after the regenerant emergence, we obtained fertile plants, which bloomed and bore fruit. The regenerants for domestic varieties of *P. ixocarpa* (cv. Likhtaryk) and *P. pubescens* (cv. Zarynka) were obtained for the first time. We established a direct relationship between the concentration of zeatin and both the frequency of plant regeneration and the number of regenerants per explant.

Key words: *Physalis peruviana, Physalis ixocarpa*, *Physalis pubescens, in vitro, regeneration, zeatin.*

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Introduction

Physalis peruviana L., *P. ixocarpa* Broth., and *P. pubescens* L. are cultivated mainly in tropical and subtropical countries. One of the main useful component of various *Physalis* species is betulin, which has antitumor properties.

In Ukraine, the above-mentioned species are grown in botanical gardens and private collections. *Physalis* is a promising plant for obtaining recombinant proteins for pharmaceutical use. Materials dedicated to the editing of the *P. pruinosa* genome by the CRISPR-Cas method have recently been published (Lemmon et al., 2018). *Physalis* can be used as a model object to study the functioning of heterologous genes in its tissues and organs. Currently, sufficient amount of works on obtaining callus tissue and regeneration of various *Physalis* species have been conducted. A study of regenerative capacity was performed by a group headed by Rao (Rao et al., 2004), which resulted in obtaining regenerants for *P. pubescens*. Initially, callus tissue was grown from leaves and internodes, and then, the regenerants on a medium MS_{30} + 2 mg/l BAP + 0.5 mg/l NAA and on MS_{30} + 2.5 mg/l BAP + 0.5 mg/l NAA were obtained from it. K. Ramar and V. Ayyadurai investigated *Physalis maxima* regeneration capacity (Ramar, Ayyadurai, 2014). H. Sandhya and R. Srinath obtained regenerants from nodal segments of *Physalis minima* (Sandhya, Srinath, 2015). K. Ramar with a group of scientists investigated

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regeneration capacity of *Physalis peruviana.* They obtained positive results of nodal segments and internode regeneration on the medium MS_{30} + 1.5 mg/l BAP + 0.5 mg/l gibberellic acid (GA_3) + 0.5 mg/l 2,4-D; MS_{30} + 2 mg/l BAP + 1 mg/l GA_3 + 1 mg/l 2,4-D, and that of leaf explants on MS_{30} + 2.5 mg/l BAP + 1 mg/l GA_3 + 0.5 mg/l 2.4-D; MS_{30} + 3 mg/l BAP + 1 mg/l GA_3 + 1 mg/l 2,4-D (Ramar et al., 2014). K. Bergier with colleagues obtained *Physalis ixocorpa* regenerants from the "hairy root's culture" on the medium MS_{30} + 5 μm Kin + 1 μm BAP (Bergier et al., 2012). O. Kumar with a group obtained regenerants of *Physalis angulata* from meristems (Kumar et al., 2015). K. Swartwood and J. Van Eck received regenerants of *Physalis pruinosa* from hypocotyls explants (Swartwood, Van Eck, 2019). N. Assad-García obtained regenerants from the cotyledons of the 12-day-old seedlings of *Physalis ixocorpa* cv. Rendidora on the MS_{30} medium + 1 μM NAA + 12.5 μM BAP (Assad-García et al., 1992). P. Singh and colleagues received regenerants from nodal segments of *P. peruviana* on the MS_{30} medium + 2.5 mg/l BAP + 0.05 mg/l indolylbutyric acid (IBA) (Singh et al., 2016). A group of researchers headed by Otroshy received regenerants of *P. peruviana* on the MS_{30} medium + 4 mg/l BAP; MS_{30} + 1 mg/l Kin + 3 mg/l BAP from leaf explants and on the MS_{30} medium +2 mg/l Kin + 2 mg/l BAP; MS_{30} + 4 mg/l BAP + 1 mg/l Kin + 0.5 mg/l indolylbutyric acid (IBA) from nodular explants (Otroshy et al., 2013). Yaroshko and Kuchuk obtained regenerants of *Physalis peruviana* (Yaroshko, Kuchuk, 2019). Several scientific groups worked with *Physalis minima* (Afroz et al., 2009; Gupta, 1986; Sheeba et al., 2015; Mungole et al., 2011; Patel et al., 1987). Despite the achievements on this approach, there is still no works on the regeneration of domestic varieties of physalis *in vitro*.

Our objective was to find an efficient culture medium for the *P. peruviana, P. ixocarpa*, and *P. pubescens* regeneration *in vitro* in order to future obtaining adult plants from the regenerants *ex vitro*.

Materials and methods

The following species were used as plant material for investigations: *Physalis peruviana, Physalis ixocarpa* (cv. Likhtaryk), and *P. pubescens* (cv. Zarynka). The originator of Likhtaryk and Zarynka varieties is M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine. The source material was taken from the collection fund of the department of cultural flora of the above-mentioned institution (Rakhmetov et al., 2015).

Seeds of the three investigated species germinated on the sterile nutrient agar medium Murashige and Skoog (MS_{30}) (Murashige, Skoog, 1962) with 30 g/l sucrose under conditions of 22–26°C, 14-hour light period, and illumination of 3000–4500 lx.

For regeneration, we used leaf cotyledons from the seven-day seedlings. The explants were cultivated horizontally one month on the MS_{30} medium, containing 30 g/l sucrose (pH 5.7–5.9) with the addition of zeatin (Zea) (assay > 98 %, Duchefa Biochemie B.V.: Netherlands) in different concentrations (0.5, 1.0, 2.0, and 3.0 mg/l).

The obtained shortened shoots were separated and transferred to the MS_{30} medium with 1 mg/l of BAP (assay > 99 %, Duchefa Biochemie B.V.: Netherlands) + 0.1 mg/l NAA (assay > 98 %, Duchefa Biochemie B.V.: Netherlands) for a month for elongating and rooting.

Data collection and Statistical analysis.

The efficacy of the used concentrations of growth stimulants for obtaining regenerants of species and varieties of the genus *Physalis* was determined by the following indicators: the number of regenerants obtained per one explant and the percentage of regeneration (regeneration frequency).

The number of regenerants was defined as a number of new young plants emerged from one explant. The regeneration frequency was calculated as a proportion (%) of the number of regenerated explants out of the total number of explants at the beginning of the experiment. The higher the percentage of regeneration was and the more regenerants were obtained from one explant, the more effective the concentration of growth stimulants used is considered.

Ten explants were used in each variant of experiments that was conducted in three replications. The data were analyzed using the general procedure in the Software Package STATISTICA Version 12. Spearman's test and standard error were used for statistical processing of the obtained data; the procedure was described in detail in our previous work (Yaroshko, Kuchuk, 2019). In this work, the effect of different concentrations of growth regulator zeatin was compared with the appearance of different numbers of regenerants per one explant.

Results and discussion

After cultivation of the explants on the MS_{30} medium with different Zea concentrations regenerants were obtained (Fig. 1, 2). The most effective medium for regeneration from leaf cotyledons was MS_{30}

supplemented with 2mg/l Zea (Fig. 2, Table 1). Quite promising results of shoot regeneration were obtained on the medium MS_{30} + 1mg/l Zea (Fig. 1, 2, Table 1). Three months after receiving regenerants, we obtained adult fertile plants, which bloomed and fruited (Fig. 3).

The regeneration of *Physalis ixocarpa* (cv. Likhtaryk) on the medium MS_{30} + 2mg/l Zea was successful, 53.84 %, while on the medium MS_{30} + 1mg/l Zea it declined to 46.15 % (Fig. 2). The regeneration of *Physalis peruviana* was lower, 45 % and 38.46 %, respectively (Fig. 2).

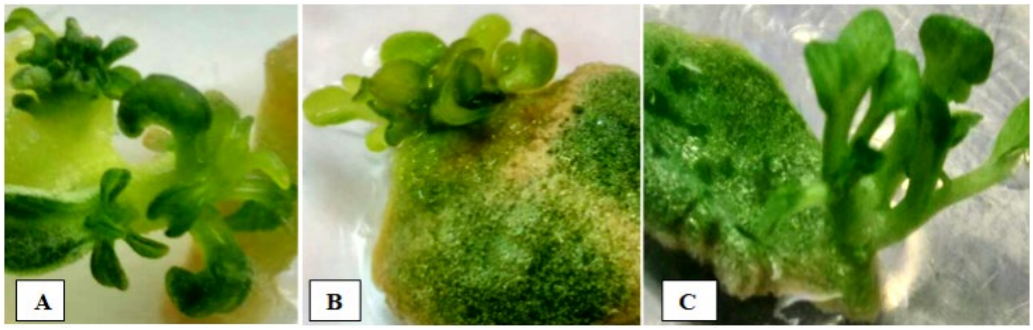

Fig. 1. Shoot induction from cotyledon leaves on the MS_{30} medium with 1mg/l Zea after one month of cultivation (A – *Physalis ixocarpa* (cv. Likhtaryk); B – *P. peruviana;* C – *P. pubescens* (cv. Zarynka))

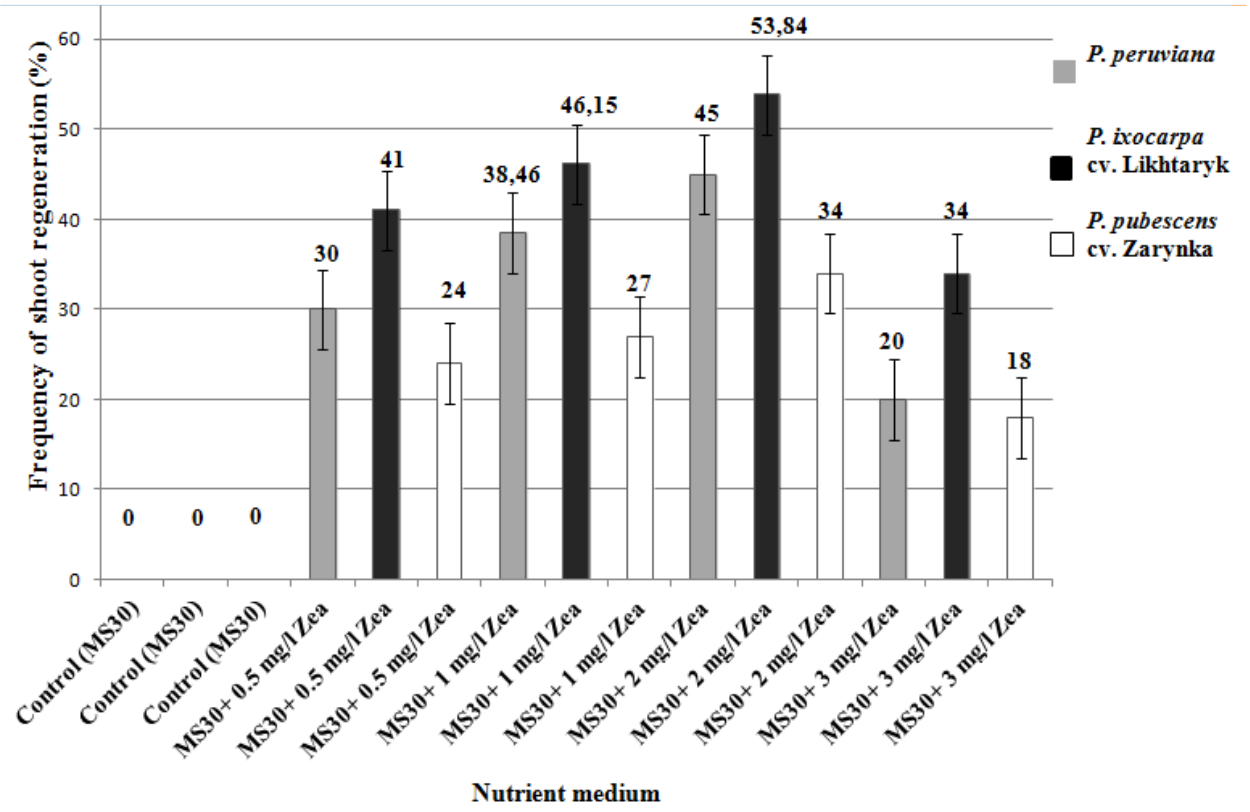

Fig. 2. Effect of zeatin on the frequency of shoot regeneration of *Physalis ixocarpa* (cv. Likhtaryk), *P. peruviana, and P. pubescens (*cv. Zarynka) from cotyledon leaves on the MS_{30} medium after one month of cultivation. Whiskers indicate the standard error; *n*=30

Table 1. Influence of growth regulator on the number of regenerated shoots from the cotyledon leaves of *Physalis peruviana, P. ixocarpa,* and *P. pubescens* on the MS_{30} medium supplemented with different Zea concentrations (number of shoots per one explant, pc., M±SE)

Plant species, variety	Concentration of Zea growth stimulant				
	0 mg/l	0.5 mg/l	1 mg/l	2 mg/l	3 mg/l
P. peruviana	-	5±0.92*	11±0.95**	14±0.96**	6±0.86*
P. ixocarpa cv. Likhtaryk	-	7±0.84*	12±1,1*	15±1.3**	10±0.83*
P. pubescens cv. Zarynka	-	3±0.63*	6±0.56**	8±0.62**	3±0.54*

** null hypothesis is rejected with significant (P≤0.05) levels of averages differences;*
*** null hypothesis is rejected with highly significant (P≤0.01) levels of averages differences.*

Fig. 3. General view of adult plants in the open ground with unripe fruits (A – *Physalis ixocarpa* (cv. Likhtaryk), B – *P. peruviana*, C – *P. pubescens* (cv. Zarynka)) and general view of ripe fruits in comparison (D, from left to right: *P. ixocarpa* (cv. Likhtaryk), *P. peruviana, P. pubescens* (cv. Zarynka)

Our data are consistent with other studies on the *Physalis* regenerative capacity. The majority of works were conducted with the use of BAP and Kin growth regulators and an addition of a third component (Ramar, Ayyadurai, 2014; Ramar et al., 2014; Kumar et al., 2015; Gupta, 1986). We used only one regulator, zeatin, and received positive results.

In our previous work, we obtained regenerants for *P. peruviana* on the media MS_{30}+ 1 mg/l kinetin (Kin) + 3 mg/l BAP and MS_{30} + 2 mg/l Kin + 1 mg/l BAP (33.33 % of regeneration on both) (Yaroshko, Kuchuk, 2019). In the current work, we got higher percentages of regeneration of the same species on the media MS_{30} + 1mg/l Zea and MS_{30} + 2mg/l Zea (38.46 % and 45 %). Thus, we can state that the MS_{30} media with Zea are more effective for obtaining *Physalis* regeneration than that with Kin or BAP.

According to the works of other researchers, the highest frequency of *P. peruviana* regeneration was obtained on the media with addition of BAP (concentration 1–3 mg/l) or Kin (1 mg/l) (Ramar, Ayyadurai, 2014; Bergier et al., 2012; Gupta, 1986). A number of regenerated plants averaged to 11 or 13 per one explant on the media with BAP or Kin, respectively. In our current work, we have achieved similar results on the media with 1 mg/l Zea (11 pc.) and 2 mg/l Zea (14 pc.)

In the world literature, there is one published work on the regeneration of *Physalis pubescens* (Rao et al., 2004) and two works on the agrobacterial transformation and regeneration of *Physalis ixocarpa* (Bergier et al., 2012; Assad-García et al., 1992). Unfortunately, the regeneration percentage obtained in both species is not indicated in these papers. Therefore, we cannot compare the results of our study with those of other research groups.

In the course of our investigation, we found out that *Physalis ixocarpa* (cv. Likhtaryk) has the highest regeneration capacity among the three species studied (53.84 %). Such a regeneration percentage is sufficient to carry out genetic transformation of experimental plants. Thus, in the further research on *Agrobacterium*-mediated genetic transformation of *Physalis* plants, we will use *Physalis ixocarpa* (cv. Likhtaryk) as the most promising candidate.

Conclusions

Our experiments resulted in definition of the most efficient culture media for regeneration of *Physalis peruviana, P. ixocarpa* (cv. Likhtaryk), and *P. pubescens* (cv. Zarynka): a percentage of shoot regeneration from cotyledon leaves was the highest on MS_{30} + 2mg/l of Zea and MS_{30} + 1mg/l of Zea. Then, the obtained regenerants were grown on the medium MS_{30} with 1 mg/l of BAP and 0.1 mg/l of NAA for elongating and rooting and, in a month, we got adult plants. Three month after the emergence of regenerants, the mature plants started blooming and bearing fruits.

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Вплив стимулятора росту зеатину на регенераційну здатність рослин деяких видів роду *Physalis* в культурі *in vitro*

О.М. Ярошко, Д.Б. Рахметов, М.В. Кучук

Метою роботи було виявлення ефективного культурального середовища для регенерації видів роду *Physalis* в умовах *in vitro* для їх подальшого розмноження і отримання плодоносних рослин з регенерантів в умовах *ex vitro*. Рослинним матеріалом для дослідження були такі види рослин: *Physalis peruviana* L.*, Physalis ixocarpa* Broth. (cv. Likhtaryk), *Physalis pubescens* L. (cv. Zarynka). Введення рослин у культуру та отримання регенерантів проводилися в умовах *in vitro*; укорінення дорослих рослин та отримання рослин зі зрілими плодами проводили в умовах теплиці та відкритого ґрунту (*ex vitro*). Для отримання регенерантів використовували середовище Мурасіге і Скуга ($МС_{30}$), доповнене стимулятором росту зеатином у концентрації 0,5–3 мг/л. Для подовження стебел регенерантів використовували стимулятор росту 6-бензиламінопурин (БАП), а для ініціації коренеутворення використовували стимулятор росту α-нафтилоцтову кислоту (НОК). Як показники ефекту різних концентрацій стимулятора росту зеатину на регенераційну здатність фізалісів використовували такі критерії: частоту регенерації рослин і кількість регенерантів, які регенерували з одного експланту. Після проведення серії експериментів були підібрані ефективні поживні середовища для регенерації *P. peruviana, P. ixocarpa* (cv. Likhtaryk), *P. pubescens* (cv. Zarynka). Найбільш ефективними середовищами для регенерації пагонів із сім'ядольних листкових експлантів виявились $МС_{30}$ + 1 мг/л зеатину (Зеа) і $МС_{30}$ + 2 мг/л Зеа. Регенерація на цих середовищах складала для *P. ixocarpa* (cv. Likhtaryk) – 46,15 % і 53,84 %, для *P. peruviana* – 38,46 % і 45 %, для *P. pubescens* (cv. Zarynka) – 27 % і 34 % відповідно. Отримані регенеранти відокремлювали від експлантів і переносили на середовище $МС_{30}$, доповнене 1 мг/л БАП + 0,1 мг/л НОК для росту стебел та вкорінення. Через місяць культивування на середовищі $МС_{30}$ + 1 мг/л БАП + 0,1 мг/л НУК були отримані ювенільні рослини, які були перенесені в тепличні умови для проходження адаптації, а пізніше – у відкритий ґрунт на експериментальній ділянці. Через три місяці (з моменту появи регенерантів) були отримані фертильні рослини, які квітували і плодоносили. Нами вперше були отримані регенеранти для вітчизняних сортів *P. ixocarpa* (cv. Likhtaryk), *P. pubescens* (cv. Zarynka). Знайдена пряма залежність між концентрацією зеатину і частотою регенерації рослин, а також між концентрацією зеатину і кількістю регенерантів, отриманих з одного експланта.

Ключові слова: *Physalis peruviana, Physalis ixocarpa*, *Physalis pubescens*, *in vitro, регенерація, зеатин.*

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Влияние стимулятора роста зеатина на регенерационную способность растений некоторых видов рода *Physalis* в культуре *in vitro*

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Целью работы было выявление эффективной культуральной среды для регенерации видов рода *Physalis* в условиях *in vitro* для их дальнейшего размножения и получения плодоносящих растений из регенерантов в условиях *ex vitro*. Растительным материалом для исследования были такие виды растений: *Physalis peruviana* L.*, Physalis ixocarpa* Broth. (cv. Likhtaryk), *Physalis pubescens* L. (cv. Zarynka). Введение растений в культуру и получение регенерантов проводились в условиях *in vitro*; укоренение взрослых растений и получение растений со зрелыми плодами проводили в условиях теплицы и открытого грунта (*ex vitro*). Для получения регенерантов использовали среду Мурасиге и Скуга ($МС_{30}$), дополненную стимулятором роста зеатином в концентрации 0,5–3 мг/л. Для удлинения стеблей регенерантов использовали стимулятор роста 6-бензиламинопурин (БАП), а для инициации корнеобразования – стимулятор роста α-нафтилуксусную кислоту (НУК). В качестве показателей эффекта различных концентраций стимулятора роста зеатина на регенерационную способность физалисов использовали такие критерии: частоту регенерации растений и количество регенерантов на один эксплант. После проведения серии экспериментов были подобраны эффективные питательные среды для регенерации *P. peruviana, P. ixocarpa* (cv. Likhtaryk), *P. pubescens* (cv. Zarynka). Наиболее эффективными средами для регенерации побегов из семядольных листовых эксплантов были $МС_{30}$ + 1 мг/л зеатина (Зеа) и $МС_{30}$ + 2 мг/л Зеа. Регенерация на этих средах составила для *P. ixocarpa* (cv. Likhtaryk) – 46,15 % и 53,84 %, для *P. peruviana* – 38,46 % и 45 %, для *P. pubescens* (cv. Zarynka) – 27% и 34% соответственно. Полученные регенеранты отделяли от эксплантов и переносили на среду $МС_{30}$, дополненную 1 мг/л БАП + 0,1 мг/л НУК для роста стебля и укоренения. Через месяц культивирования на среде $МС_{30}$ + 1 мг/л БАП + 0,1 мг/л НУК были получены ювенильные растения, которые были перенесены в тепличные условия для прохождения адаптации, а позже – в открытый грунт на экспериментальном участке. Через три месяца (с момента получения регенерантов) были получены фертильные растения, которые цвели и плодоносили. Нами впервые были получены регенеранты для отечественных сортов *P. ixocarpa* (cv. Likhtaryk), *P. pubescens* (cv. Zarynka). Обнаружена прямая зависимость между концентрацией зеатина и частотой регенерации растений, а также между концентрацией зеатина и количеством регенерантов, полученных от одного экспланта.

Ключевые слова: *Physalis peruviana, Physalis ixocarpa*, *Physalis pubescens*, *in vitro, регенерация, зеатин.*

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