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Influence of in vitro cultivation factors on micropropagation and alkaloid determination of Convolvulus persicus L.

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Abstract. *Convolvulus persicus* L. is a critically endangered species native to Bulgaria. Studying the conditions for its in vitro cultivation is important for its ex-situ conservation. A number of factors influence the growth, development, and multiplication of in vitro plants. In this study, the effects of several medium components - including the type of gelling agent, the quantity of macrosalts, activated charcoal supplementation, and plant growth regulators - were investigated to determine the most suitable conditions for the in vitro cultivation of the species. The effects of explant type and culture vessels were also examined. The type of gelling agent (Gelrite) and the explant significantly affected culture growth. Culture vessels and plant growth regulators had a smaller influence, while the effect of activated charcoal depended on its combination with the other factors. The quantity of macrosalts did not influence plant growth in vitro. A phytochemical analysis of the alkaloid content of *Convolvulus persicus* was conducted for the first time. The phytochemical analysis revealed that three alkaloids - nicotine, tropinone, and N-methylpyrrolidinyl-cuscohygrine - were biosynthesized by *C. persicus* at two developmental stages. Nicotine was identified as the major alkaloid in the species.

Key words: gelling agent, macrosalts, activated charcoal, culture vessels, explant type, alkaloids.

Introduction

Convolvulus persicus L. (Convolvulaceae) is a psammophytic species occurring in small, scattered local populations, with a general distribution along the coastal areas of the Caspian Sea and the Black Sea (POWO, 2025). The species is listed as critically endangered in the Red Data Book of the Republic of Bulgaria. *C. persicus* is known from only two populations in Bulgaria, located near the city of Varna and lake Durankulak. The natural habitats of *C. persicus* are threatened by both natural and anthropogenic factors, including coastal erosion, tourist pressure, sand dune destruction (Kiss & Szatmari, 2020).

Habitat modification of *C. persicus* requires both in situ and ex situ conservation measures. In vitro techniques are particularly suitable because they allow easy and rapid vegetative propagation and can be applied to species with low seed germination capacity. For example, Kiss & Jarda (2021) reported only 1% germination of *C. persicus* seeds sown in vitro.

A number of factors influence the growth, development, and multiplication of in vitro plants, including the characteristics of the culture medium. Comprehensive research is necessary in order to determine the most suitable conditions for in vitro cultivation of a given plant species. Explant type, medium composition, supplementation with activated charcoal (AC), type of culture vessels, gelling agent, and the presence of plant

growth regulators (PGRs) all influence the success of in vitro cultivation (Bouaaza et al., 2024).

The choice of an appropriate gelling agent ensures the development of healthy and vigorous plants (Bouaaza et al., 2024). Gelrite, a polysaccharide used as an alternative to agar, also facilitates the visual detection of contamination in the gelled medium (Palanyandy et al., 2020). Moreover, gel strength can be influenced by salt concentration, which can also affect shoot formation and root length (Arnold et al., 1995; Baker & Wetzstein, 1994; Castillo, 1998).

Several authors have investigated the influence of AC on plant micropropagation efficiency (Boggetti et al., 1999; Borges et al., 2001; Hazra et al., 2002; Quoirin et al., 2001). AC is often used in plant tissue cultures to improve culture establishment and plant regeneration. It can influence shoot formation, cell growth and development, somatic embryogenesis, plant recovery, and rooting (Thomas et al., 2008).

Culture vessels provide isolation from the external environment and can influence plant growth and development due to differences in volume, material, and closure type, which in turn affect gas exchange, relative humidity, and the anatomical and morphological characteristics of plants (Guatam et al., 2024; Vollmer et al., 2024).

The explant types most frequently used for micropropagation are nodal segments and axillary buds (Bouaaza et al., 2024). Together with different combinations and concentrations of auxins and cytokinins, they form the basis of in vitro culture establishment.

To date, essential oils are the only secondary metabolites that have been investigated in this species. Dehghan et al. (2015) identified 0.04% (v/w) essential oil content in *C. persicus*, with β-caryophyllene comprising 47.0% of the total. The methanolic extract of the aboveground parts of the species has been shown to possess antibacterial activity (Dehghan et al., 2020) as well as antioxidant and glucosidase-inhibitory properties (Dehghan et al., 2015).

The aim of the study was to investigate the effects of different cultivation factors - such as explant type, macrosalt concentration, gelling agents, activated charcoal, cultivation vessels, and plant growth regulators - on the in vitro multiplication rate of the species. The alkaloid content of native plants was also examined.

Materials and methods

Plant material

Mature and immature seeds were collected from the natural locality of the species near lake Durankulak, the northernmost point of the Bulgarian Black Sea coast. Aboveground plant material was collected for the phytochemical analysis. A voucher specimen was deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (SOM 167868).

Seed sterilization and germination

The seeds were surface-sterilized with 70% ethanol for 1–2 min, followed by 10 min in diluted commercial bleach (chlorine < 2.5%), and then rinsed three times with sterile distilled water. The seeds were germinated on basal B5 medium (Gamborg et al., 1968) with 20 g/L sucrose and solidified with 6.5 g/L Plant agar (Duchefa, NL). The obtained seedlings were used as a source of explants for the establishment of the in vitro culture.

In vitro cultivation

The explants were cultivated on the following media: B5; B5 supplemented with 0.5 g/L AC (B5 + AC); B5 with double concentration of macrosalts (2B5); and 2B5 supplemented with 0.5 g/L AC (2B5 + AC). All media contained 20 g/L sucrose, and the pH was adjusted to 5.75 prior to autoclaving at 121°C for 20 min.

In order to study the influence of explant type on in vitro propagation, two types of explants were examined: nodal segments with three nodes and shoot segments with an apical bud. The effect of different gelling agents on explant growth was also investigated. Gelrite (Duchefa, NL) at 2 g/L and agar (Duchefa, NL) at 6.5 g/L were used as solidifying agents. Plants were cultivated in two types of culture vessels: glass jars and plastic vessels (Sterivent high containers, Duchefa, NL).

Medium 2B5 solidified with 2 g/L Gelrite was supplemented with three combinations of NAA and the cytokinins TDZ, mT, and 2iP as follows: 0.1 mg/L TDZ + 0.01 mg/L NAA; 1 mg/L mT + 0.1 mg/L NAA; 1 mg/L 2iP + 0.1 mg/L NAA.

Their effects on the development of apical shoot explants were evaluated. Observations were performed after one and two months. Each experimental variant consisted of two replicates with ten explants per cultivation vessel. The number of axillary shoots was presented as mean number of

shoots formed per explant. All cultures were maintained in a culture room at 23 ± 2°C with a 16 h light/8 h dark photoperiod.

Extraction and identification of alkaloids

The air dried and ground plant materials at two stages of development (flowering plants - 13.4 g and mature fruits – 13.5 g) of *Convolvulus persicus* were worked up separately, following the same procedure. The plants materials were extracted exhaustively with 95% EtOH in a Soxhlet apparatus. The EtOH extracts were evaporated under reduced pressure, acidified with 3% HCl to pH 1–2 and left overnight at room temperature. The insoluble nonalkaloid substances were removed by filtration and the filtrates were subjected to three times n-hexane extraction to eliminate the rest of the nonalkaloid materials. Thus purified acidic solutions were made alkaline with 25% NH_4OH to pH 9–10 and extracted six times with CH_2Cl_2. The combined CH_2Cl_2 extracts were dried (anh. Na_2SO_4) and evaporated under reduced pressure to give crude mixtures of tertiary alkaloids as follow: from *C. persicus* - blossom stage - 10.96 mg and from *C. persicus* - mature stage - 22.95 mg. The crude mixtures of alkaloids were analyzed by GC-MS (GC-MS: Hewlett Packard 6890/MSD5973 instrument, HP-5 MS column (30 m x 0.25 mm x 0.25 µm), temperature program: 50 to 300°C at 4°C min^{-1} and 10 min hold at 300°C, injector temperature - 280°C, flow rate of carrier gas (He) - 0.8 ml min^{-1}) and three alkaloids were detected (Table 1). Alkaloid mass spectral data were compared with those in the literature (El-Shazly & Wink, 2008) and electron ionisation mass spectral library Wiley 275®.

Statistical analysis

SigmaStat v.14.0 was used for the statistical analyses. The Shapiro–Wilk normality test was applied to assess data distribution. Statistical significance was evaluated using the Mann–Whitney rank-sum test and Student's t-test at $p \leq 0.05$. Results are presented as mean values of three replications ± standard deviation (SD). Values marked with different letters in the figures indicate statistically significant differences.

Results

Explant type and macrosalt quantity

The growth of *C. persicus* was better in apical shoot cultures than in nodal explant cultures, with both plant length and bud formation rate were higher (Fig. 1 and Fig. 2). The growth of nodal explants was slower than that of explants with apical bud. No significant differences in plant length were observed among the different media types. However, the development of the nodal explants was still retarded.

The studied parameters were highest on media 2B5 (2B5 and 2B5 + AC). However, different macrosalt quantities did not significantly influence explant development, their length, or bud formation.

Influence of activated charcoal

It was found that the influence of AC depended on its combination with the other factors studied. Supplementation of the media with AC led to a slight increase in the number of adventitious shoots in the apical shoot cultures, but not in length of the plants (Fig. 1 and Fig. 2). The opposite effect was observed in nodal explant cultures, where both the number of adventitious shoots and the length of the plants were higher in media without AC.

Influence of the gelling agent

It was found that the type of gelling agent had a strong influence on the development of the explants. The length of apical shoots cultured on medium gelled with Gelrite was, on average, 2.5 times greater than that of explants grown on media solidified with agar (Fig. 3). In all examined variants (different macrosalt concentrations, with or without AC), the length of the plants grown on Gelrite-gelled media was higher than that on media gelled with agar. No statistically significant difference was observed between agar- and Gelrite-solidified media in terms of shoot formation. In both cases, explants produced an average of two axillary shoots.

Plant growth in two types of culture vessels

Plants cultured in plastic vessels developed more rapidly and attained significantly greater length than those grown in glass jars (Fig. 4.). Plants grown in jars produced, on average, a higher number of adventitious shoots (three shoots per explant). Additionally, plants cultivated in jars were longer when grown on medium 2B5 and AC did not affect plant length when supplemented into the media.

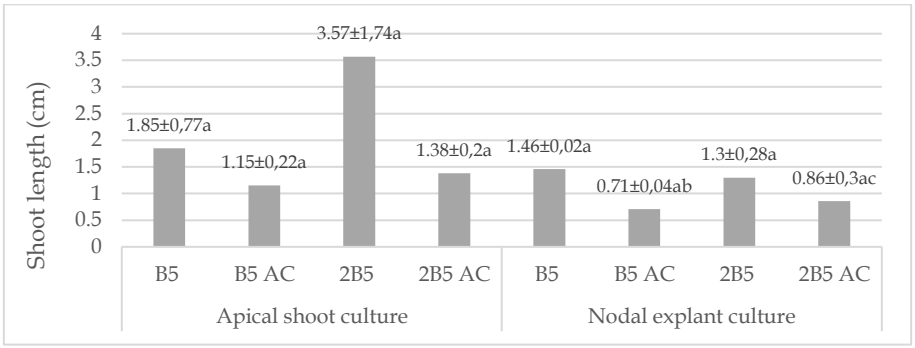


Fig. 1. Effects of activated charcoal (media B5 AC and 2B5 AC) and macrosalt concentrations (B5 and 2B5) on shoot length in apical shoot (cm) and nodal explant cultures. Different letters show statistically significant difference.

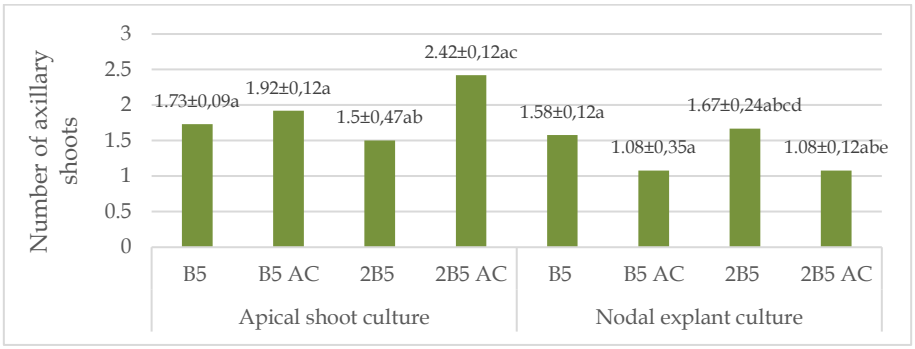


Fig. 2. Effects of activated charcoal (media B5 AC and 2B5 AC) and macrosalt concentrations (B5 and 2B5) on shoot formation in apical shoot and nodal explant cultures. Different letters show statistically significant difference.

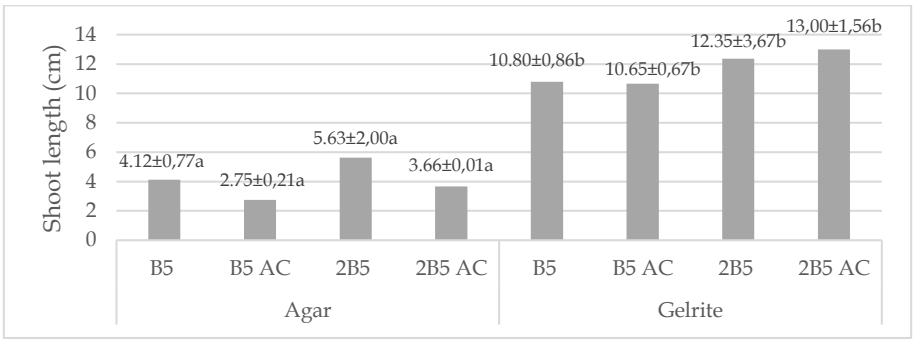


Fig. 3. Influence of the type of gelling agent (Agar and Gelrite) on shoot length (cm). Different letters show statistically significant difference.

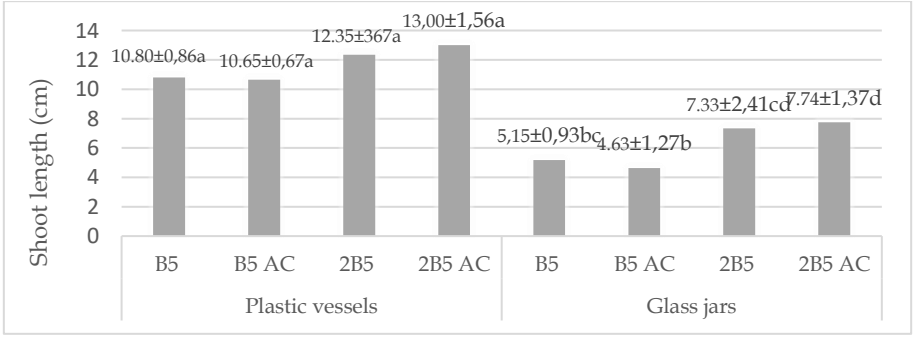


Fig. 4. Effect of the type of culture vessels (plastic vessels and glass jars) on shoot length (cm). Different letters show statistically significant difference.

Influence of plant growth regulators

PGR did not stimulate significantly shoot elongation after one month of cultivation (Fig. 5). Moreover, the presence of 2iP retarded plant growth and adventitious shoot formation. During the second month, growth in the control variant was reduced, whereas growth in the PGR-supplemented variants accelerated, which partially compensated the difference between them and the control.

A high number of shoots was formed in the control variant, and supplementation with PGR did not stimulate adventitious shoot formation (Fig. 6). In fact, in this case, 2iP even inhibited shoot formation. PGR mT stimulated shoot formation after two months of cultivation in jars.

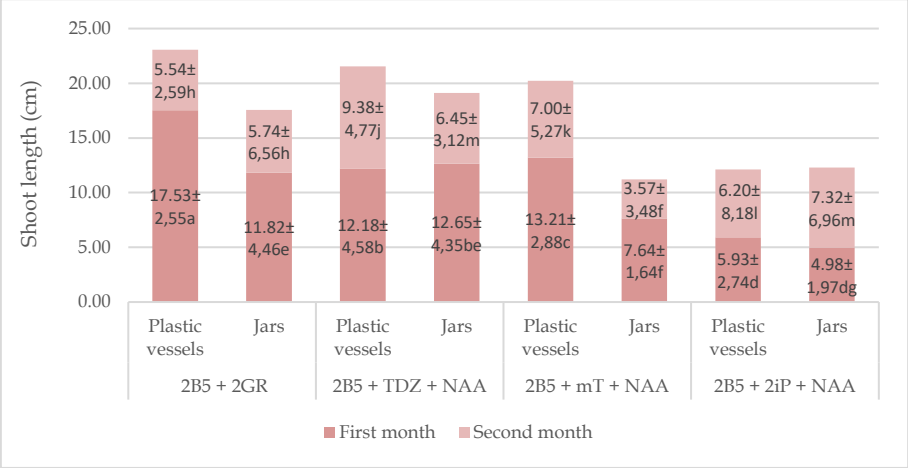


Fig. 5. Influence of PGRs (TDZ; mT; 2iP; NAA) on shoot length (cm) of the cultures in two consecutive months. Different letters show statistically significant difference.

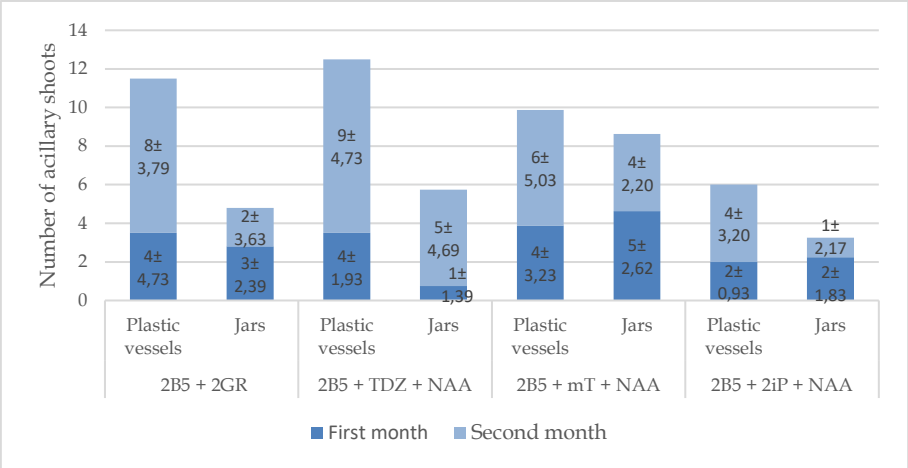


Fig. 6. Influence of PGRs (TDZ; mT; 2iP; NAA) on shoot formation in two consecutive months. Different letters show statistically significant difference.

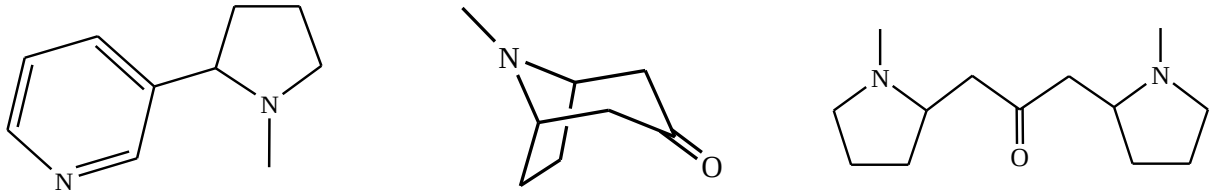
Phytochemical research on C. persicus

The alkaloid profile of *C. persicus* was analyzed by GC/MS during two developmental stages: the flowering stage and the maturity stage. Three alkaloids were identified - nicotine (1), tropinone (2), and N-methylpyrrolidinyl-cuscohygrine (3) (Table 1 and Fig. 7). The results showed that both developmental phases exhibited a similar alkaloid composition, with 1 being the predominant compound. The only difference observed was the presence of 3 in the maturity stage, which was absent during the flowering stage.

Table 1. Alkaloids identified in *C. persicus* in two stages of development.

Alkaloids	Blossom stage	Mature stage	MS references
Nicotine (**1**)	++	++	Wiley 275®
Tropinone (**2**)	+	+	Wiley 275®
N-Methylpyrrolidinyl-cuscohygrine A (**3**)	-	+	El-Shazly & Wink (2008)

Legend: "++" main alkaloid; "+" presence; "-" absence



Nicotine (**1**) Tropinone (**2**) N-Methylpyrrolidinyl-cuscohygrine A (**3**)

Fig. 7. Structural formulas of the identified alkaloids.

Discussion

Our research showed that cultures of *C. persicus* were affected by the cultivation factors to different degrees. One of the most important factors influencing the chemical and physical characteristics of culture media is the type and concentration of the gelling agent (Bouaaza et al., 2024; Palanyandy et al., 2020). This was also confirmed by our results for the cultivation of *C. persicus*. Among all factors examined, the gelling agent had the strongest effect on the growth of *C. persicus*.

The type of gelling agent can significantly influence the growth and development of cultivated plants. According to Al-Mayahi & Ali (2021), Gelrite increases bud formation, reduces medium browning, and eliminates shoot vitrification. Shoot growth and adventitious shoot formation were also reported to be higher when Gelrite was used for the cultivation of various plant species, including banana (Buah et al., 1999), apple (Welander & Maheswaran, 1992), *Ranunculus* sp. (Beruto et al., 1999), and the slow-growing cultivar *Solanum tuberosum* cv. Baraka (Veramendi, 1997).

Gelrite is particularly effective in the conversion of somatic embryos, with similar results reported for *Elaeis guineensis* (Palanyandy et al., 2020), *Papaver degenii* (Doycheva et al., 2022), and *Picea* sp. (Tremblay & Tremblay, 1991).

When Gelrite was combined with some of the other tested factors, the differences among variants became smaller and statistically insignificant, indicating the decisive role of Gelrite in the cultivation of *C. persicus*.

The better results obtained in cultures grown on media solidified with Gelrite can be attributed to the strength of the gel and the improved availability of nutrients (Sholten & Pierik, 1998), as the type of gelling agent is known to influence both the concentration and the availability of mineral nutrients (Buah et al., 1999; Singha, 1984; Kusumoto, 1980). The gelling agent modifies the properties of the medium by affecting diffusion, availability, and uptake of mineral nutrients and water (Buah et al., 1999). As a result, morphogenesis, growth, and plant development are influenced (Bouaaza et al., 2024). In this regard, the control variant and the variant supplemented with TDZ and NAA, both cultivated on media gelled with Gelrite, showed the best results in terms of shoot length and adventitious shoot formation.

Weaker growth and reduced shoot formation were observed when agar was used as a gelling agent in plastic vessels compared with the combinations Gelrite + Plastic vessels and Gelrite + Jars. This confirmed the stimulatory effect of Gelrite on *C. persicus* regardless of the type of cultivation vessel (Agar + Plastic vessels and Gelrite + Jars).

The type of cultivation vessel and the type of lid influence gas composition and light penetration within the vessels. In addition, they protect

the medium and the cultures from contamination and excessive water evaporation (Kaçar et al., 2010). The degree of light penetration and the humidity within the vessels may explain the differences observed between the two culture systems (Gelrite + Plastic vessels and Gelrite + Jars).

AC is used for various purposes, including adsorption of exudates, impurities, and inhibitory substances, as well as for improving the growth and development of plant tissues (Thomas, 2008). Its effect is attributed to reducing the accumulation of exudates in the medium. The influence of AC on *C. persicus* was ambiguous and depended on its interaction with other factors such as explant type, gelling agent and culture vessels. AC did not inhibit explant growth on Gelrite-solidified media.

The phytochemical study of the alkaloid content of *C. persicus* was conducted for the first time, leading to the identification of three alkaloids: nicotine (1), tropinone (2), and N-methylpyrrolidinyl-cuscohygrine (3). Nicotine (1) is reported for the first time in the genus *Convolvulus*. Although nicotine has been detected in more than 60% of the species within the family Convolvulaceae - across almost all tribes, genera, and even sections of large genera - it generally occurs as a minor component (Eich, 2008). In contrast, in *C. persicus*, nicotine represents as a predominant alkaloid in the mixture during both developmental stages of the plant (Table 1). Further studies are required to determine whether this alkaloid is synthesized as a genuine secondary metabolite of the plant or as a result from allelopathic interactions.

Conclusions

The results showed that Gelrite and the type of explant significantly affected the growth of *C. persicus* in vitro cultures. The growth of the cultures depended strongly on the explant type. Culture growth was influenced to a lesser extent by PRGs and culture vessels. The effect of AC depended on its combination with other factors. It was also observed that the quantity of macrosalts did not affect the in vitro growth of the plants. For the first time, three alkaloids - nicotine, tropinone, and N-methylpyrrolidinyl-cuscohygrine - were identified in *C. persicus*. Nicotine was found to be the main alkaloid in the species.

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