

Research

“Biotechnological approaches for conservation of the vulnerable *Capparis spinosa* var. *aegyptia*”

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Abstract

Saint Katherine Protectorate, one of the largest protected areas in Egypt, is home to a variety of rare and endangered species. Among them is *Capparis spinosa* var. *aegyptia*, a plant that is facing population decline due to two key factors; over-harvesting for traditional medicine and drought-induced stress. DNA barcoding analysis and in vitro culture techniques were conducted in this research for the first time to save this endangered variety, aligning with the United Nations Sustainable Development Goal 15 for ecosystem preservation and biodiversity conservation. PCR amplification and sequencing, utilizing DNA markers *trnH-psbA*, maturase K (*matK*), and ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), were used to authenticate the plant. BLAST results and phylogenetic analysis confirmed the identification of the plant. In vitro propagation of the plant was achieved by culturing stem nodal segments on Murashige and Skoog (MS) medium supplemented with various cytokinins and 0.5 mg/l of α -Naphthalene acetic acid (NAA). To enhance shoot multiplication, different cytokinin combinations were evaluated. The highest growth induction (95%) and mean number of shoots (11.2) were observed with MS medium containing 1 mg/l each of 6-benzyl adenine (BA) and kinetin (Kin). Multiple shoots were successfully rooted on quarter-strength ($\frac{1}{4}$) MS medium fortified with 3.0 mg/l NAA. After rooting, the plants were transferred to pots containing a mixture of sand, soil, and peat moss, and acclimatized in a greenhouse with a survival rate of 95%. This study demonstrates the potential of in vitro culture as a short-term conservation technique to propagate the endangered *C. spinosa* var. *aegyptia*, and highlights the importance of preserving and restoring our terrestrial ecosystems for sustainable development.

Article Highlights

- Two successful methods were developed to protect and conserve *C. spinosa* var. *aegyptia* from extinction.
- DNA barcoding ensured accurate plant identification, aiding in its precise classification and conservation.
- An efficient in vitro propagation protocol increased plant production, helping support biodiversity and ecosystem restoration.

Keywords Capparaceae · DNA barcoding · Micropropagation · Southern Sinai

Abbreviations

ANOVA Analysis of variance

BA 6-Benzyl adenine

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BLAST	Basic local alignment tool
CBOL	Consortium for the barcode of life
CpDNA	Chloroplast DNA
IBA	lindole-3-butyric acid
2Ip	N6-(2-isopentenyl) adenine
Kin	Kinetin
<i>matK</i>	Maturase K
MS	Murashige and Skoog
NAA	α -Naphthaleneacetic acid
NCBI	National Center of Biotechnology Information
PGRs	Plant growth regulators
<i>rbcl</i>	Ribulose-1,5- biphosphate carboxylase/oxygenase large subunit
SD	Standard Deviation
TDZ	Thidiazuron

1 Introduction

Plants have long been recognized as a valuable source of therapeutic agents, and a significant proportion of modern drugs are derived from plants or their derivatives [1]. With over 70,000 species of plants used for medicinal purposes, it is crucial for the scientific community and decision-makers to identify locally available plants. Doing so would help ensure continued access to the many benefits these plants have to offer [2]. *Capparis spinosa* consists of four varieties in Egypt, including *C. spinosa* var. *aegyptia* (Lam.) Boiss. *C. spinosa* var. *aegyptia* belongs to the Capparaceae family and is commonly known as Caper-bush in English and Kebbar, Lasaf or Laisouf in Arabic [3–5]. The plant is distributed worldwide and is found in the Arabian Peninsula, Northern Africa, Southern Europe, and the Mediterranean [6]. The fruit and buds of the plant are edible [7] and it is known for its medicinal and economic value. *C. spinosa* is rich in metabolites such as alkaloids, flavonoids, polyphenols, lipids, glucosinolates, indoles, and terpenes [8]. The caper plant has been found to possess numerous therapeutic benefits, including its antifungal, antibacterial, antioxidant, antiallergic, antidiabetic, anti-inflammatory, antihyperlipidemic and anticancer properties. It is also known to be hepatoprotective and anthelmintic [9]. The plant is safe to use, and there have been no reported harmful effects from its use [10]. Its fruits are particularly useful in the treatment of diabetes, hypertension, fever, and rheumatism, while the buds, roots, and leaves are effective in treating earache, gastric, dermatological, kidney, and liver disorders, as well as headache and tooth pain [8, 11, 12]. The Caper plant is also widely used in the cosmetics industry, in addition to being a popular flavouring in cooking [13, 14].

C. spinosa is a valuable medicinal species currently facing several global and local threats, including drought, overgrazing, human activity, and overcollection for trade and domestic use, which have caused a rapid population decline [15, 16]. In Egypt, it was listed as vulnerable in 2000 due to overexploitation, leading to its increasing rarity. Despite its importance in traditional medicine and consumption, the species has not been reassessed since 2000, and conservation efforts through in situ and ex situ methods are urgently needed to protect the remaining populations. [5, 6].

The conservation of endangered and wild plants is vital for safeguarding biodiversity, as these species play crucial roles in maintaining ecosystem balance, supporting wildlife, and providing genetic diversity. Protecting them ensures the preservation of valuable resources for medicine, agriculture, and environmental resilience. Additionally, conserving these plants helps combat the loss of unique species and habitats, contributing to global efforts aimed at sustaining biodiversity and promoting ecosystem health for future generations [17].

Molecular identification of plant species through DNA barcoding is a crucial step in botanical research and ex situ conservation efforts. By using a universal short DNA sequence, researchers can accurately identify plant species, even in cases where traditional morphological identification methods are challenging [18, 19].

The Consortium for the Barcode of Life's Plant Working Group (CBOL) recommended *rbcl* and *matK* as the standard barcode regions, while also suggesting *trnH-psbA* as complementary loci. Unlike traditional taxonomic methods, which typically require a full plant, ideally in bloom, for accurate identification, DNA barcoding offers the ability to identify species using only small tissue samples. This makes it a valuable tool for agencies tasked with combating illegal trade and biopiracy. One key application of DNA barcoding in conservation is identifying endangered species involved in illegal trade, even from partial or vegetative samples [20].

In vitro propagation is another valuable technique that allows for the mass production of plants for ex situ conservation and commercial purposes [21, 22]. In vitro propagation has been identified as a potential solution, as studies have shown that plants display a wide range of response to in vitro propagation ability not only among families and species but also within genotypes in the same species [23, 24]. Combining these two techniques can have significant implications for the conservation and sustainable use of plant species. For example, in vitro propagation of endangered plant species can help preserve their genetic diversity, while DNA barcoding can be used to verify the identity of the propagated plants [25].

The conventional methods of propagating *C. spinosa* have not been successful due to poor rooting percentages in stem cuttings and inhibitors in the seed coat that reduce germination rates in seed propagation. Moreover, the species exhibits a high level of seed heterozygosity, resulting in genetic variability among offspring [14, 26]. To overcome these challenges, vegetative propagation techniques present a valuable alternative. These methods ensure greater uniformity and consistency in plant production, as they produce genetically identical plants, preserving the specific traits of the parent plant. In contrast, sexual propagation often leads to variability, making it difficult to maintain desirable characteristics across generations. Additionally, vegetative techniques offer more efficient and reliable means of propagation by bypassing the low rooting success seen in traditional methods. Therefore, developing effective in vitro propagation protocols is crucial for uniform, large-scale production of *C. spinosa*, ensuring compliance with quality standards and preserving essential traits for both research and commercial use [27].

To date, few notable studies from around the world have conducted micropropagation protocols on different varieties of *C. spinosa* L. grown in various habitats except var. *aegyptia*. Previous studies on the micropropagation of *C. spinosa* have employed various methods, nutrient media, and growth regulators to optimize in vitro propagation. These studies include both direct and indirect approaches for micropropagation. Examples of such studies include those conducted by Musallam et al. [16], Carra et al. [28], Elmaghrabi et al. [12], Attia et al. [29] and Gianguzzi et al. [14]. In Egypt, El-Mekawy et al. [13] and Hassan and AbdelKader [26] reported the in vitro propagation of a wild variety of *C. spinosa* found in North Sinai. While several studies have been conducted on different varieties of *C. spinosa*, there is a need for further research on the variety *aegyptia* found in Southern Sinai, Egypt. Therefore, this study aims to authenticate the variety *aegyptia* of *C. spinosa* using DNA marker technologies, including barcoding, as part of a conservation strategy. Additionally, in vitro propagation was employed to produce and multiply this variety as a genotype growing in Saint Katherine, Southern Sinai. Combining in vitro propagation with DNA barcoding offers an effective ex situ conservation approach to protect this endangered plant.

2 Materials and methods

2.1 Plant collection and traditional identification

The wild plant samples of *C. spinosa* var. *aegyptia* (Fig. 1a, b) were collected from Wadi Zaghra, Saint Katherine Protectorate, South Sinai (N: 28.63041 E: 34.12809) (Fig. 2) under the guidance of the reserve authorities, adhering to the minimum sample collection requirements. The plant samples were identified with the help of the expert taxonomists in the field. Target plant species was identified based on Boulos [3], then collected samples were compared with well identified herbarium specimens kept in Saint Katherine Protectorate and Faculty of Science herbaria. The taxon name was reconciled against Kew's Plants of the World Online [30] and International Plant Names Index (<https://www.ipni.org/>). The plant specimens were deposited at the Herbarium of Faculty of Science, Cairo University with the voucher number of 35.116.326.

2.2 Molecular identification by DNA barcode analysis

2.2.1 DNA extraction

The genomic DNA of *C. spinosa* var. *aegyptia* was extracted from 100 mg leaves using CTAB protocol [31]. NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc.) was used to quantify the DNA concentration. PCR amplification was conducted using 10 ng/μl of DNA.

Fig. 1 **a.** *C. spinosa* var. *aegyptia* plant growing naturally in Wadi Zaghra, Saint Katherine, Southern Sinai. **b.** a close-up view of a flower

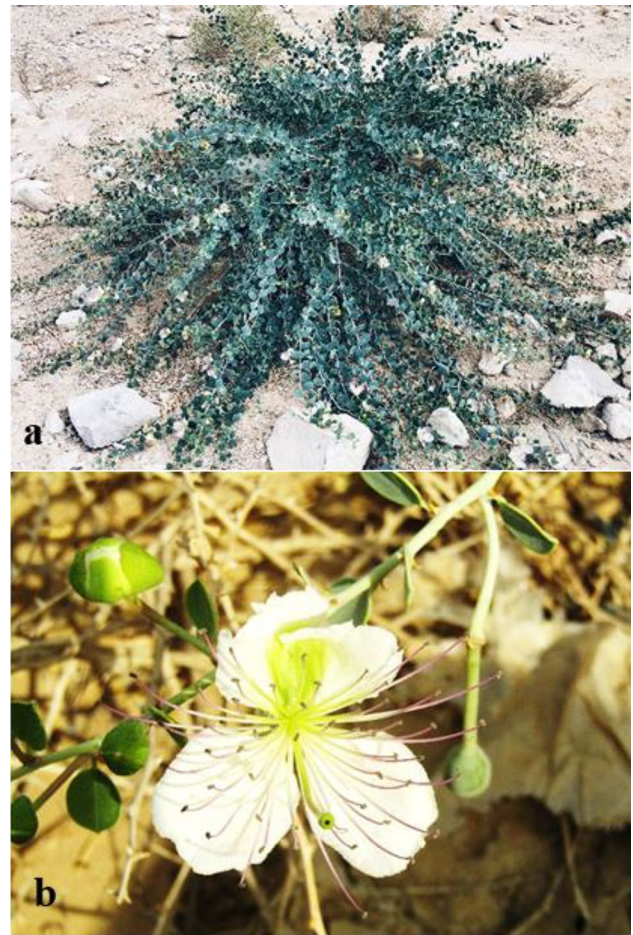
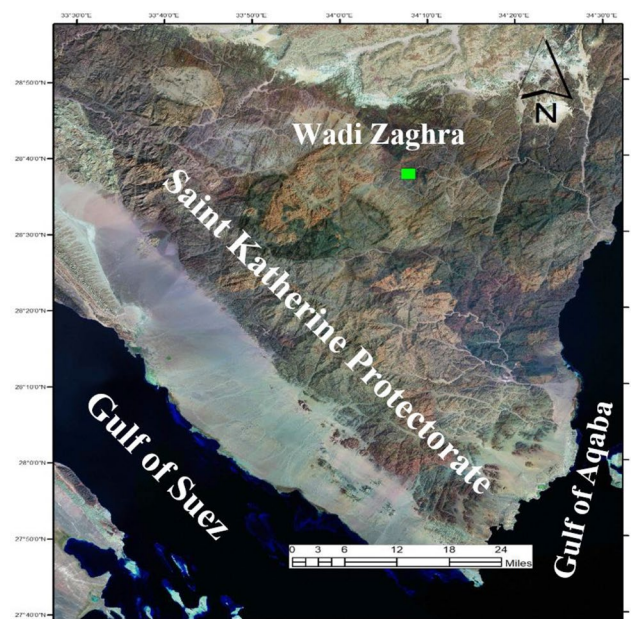


Fig. 2 A map showing the location of Wadi Zaghra, the collection site of *C. spinosa* var. *aegyptia*



2.2.2 DNA barcodes PCR analysis

The PCR amplification was conducted as reported by Ibrahim et al. [32]. for the three chloroplast DNA barcodes; *psbA-trnH*, *matK* and *rbcL* in a Thermal Cycler TC-TE BOE 8, 089, 6022 (BOECO, Germany). Table 1 lists the sequences of the three primer pairs and their loci that were used. The PCR reaction was adjusted to a total reaction volume of 50 µl that contains about 40 ng genomic DNA, ThermoScientific Dream Taq Green PCR Master Mix (2X), 200 Reactions (Catalog No: K1081), 1.5 mL of each primer (2.5 mM), and well purified water to the final volume. To perform the PCR reaction, a total of 35 cycles were completed after an initial denaturation cycle of 4 min at 95 °C. During each cycle, there was a denaturation phase lasting 30 s at 94 °C, followed by an annealing phase of 30 s at 57 °C and an elongation phase of 50 s at 72 °C. The final cycle included a primer extension segment that was prolonged to 10 min at 72 °C.

The PCR amplicons obtained were analyzed through electrophoresis, using a 1.5% agarose gel that contained ethidium bromide (0.5 µg/mL). The gel electrophoresis was conducted with 1X TAE buffer. To determine their molecular size, a GeneRuler 1 Kb plus DNA ladder (Thermo Scientific Catalog number: SM1322) was utilized. The PCR-generated products were examined using a UV transilluminator. After identification of the bands with the appropriate molecular size, they were purified from the agarose gel using the Gene JET Gel Extraction Kit (Thermo Scientific Catalog num: K 0691). The purified products were sent to the MacroGen laboratory located in Seoul, South Korea for sequencing analysis.

2.2.3 Sequence data analysis

Once the sequences were obtainable, the peripheral noisy parts at the 5' and 3' sequences of the three barcodes (*psbA-trnH*, *matK* and *rbcL*) were removed. By using the default parameters, the cleaned sequences were used to classify the plant through comparing with previously published sequences using Basic Local Alignment Search Tool nucleotide (BLASTn) available at the National Centre of Biotechnology Information (NCBI) database. At last, the NCBI team sent the accession numbers of the three barcode sequences after being submitted to the GenBank databases. The Phylogenetic trees were constructed using the free online tool Clust Omega ([https:// www.ebi.ac.uk/Tools/msa/clustalo/](https://www.ebi.ac.uk/Tools/msa/clustalo/)).

2.3 In vitro propagation

2.3.1 Explants preparation

The *C. spinosa* var. *aegyptia* shoots that were collected were cleaned using water with 2–3 drops of Tween-20 for a duration of 15 min to get rid of any dirt. They were then separated into pieces that were roughly 4 cm in length and washed with running water for approximately 25 min. To ensure that they were aseptic, the explants were initially disinfected by immersing them in a freshly prepared 0.1% (w/v) mercury chloride (HgCl₂) solution for one minute under a laminar airflow cabinet. Subsequently, they were washed three times with distilled sterilized water and sterilized with 1.3% (w/v) sodium hypochlorite (NaOCl) for 20 min while being gently stirred. Afterward, the sterilized stem nodal segments were washed thoroughly four to five times with deionized water to eliminate any NaOCl residue before being cut into stem nodal segments with two nodes. Finally, the small parts were transferred to culture media in aseptic manner.

2.3.2 Culture media and growth condition

The aseptic stem nodal segments were cultivated in Murashige and Skoog (MS) [36] synthetic medium containing 3% sucrose (w/v), along with various concentrations and combinations of plant growth regulators. The pH of the medium

Table 1 List of DNA barcoding loci; *trnH-psbA*, *matK* and *rbcL* regions and the primer pairs used in the PCR reactions

Primer name		Sequence 5'- 3'	Reference
<i>trnH-psbA</i>	<i>psbA3_f</i>	GTT ATG CAT GAA CGT AAT GCT C	[33]
	<i>trnHf_05r</i>	CGC GCA TGG TGG ATT CAC AAT CC	
<i>matK</i>	390 F	CGA TCT ATT CAT TCA ATA TTT C	[34]
	1326 R	TCT AGC ACA CGA AAG TCG AAG T	
<i>rbcL</i>	1 F	ATG TCA CCA CAA ACA GAA AC	[35]
	724 R	TCG CAT GTA CCT GCA GTA GC	

was adjusted to 5.7 ± 0.1 prior to the addition of 0.3% (w/v) phytagel for solidification of the medium. The medium was poured into jars and then sterilized via autoclaving at a pressure of 1.06 kg/cm and a temperature of 121 °C for 20 min. The cultures were maintained under a 16-h photoperiod of cool fluorescent light with a light intensity of 2500–3000 lx (F140t9d/38, Toshiba) at a constant temperature of 26 ± 1 °C and 60–70% relative humidity.

2.3.3 Influence of various cytokinin types and concentrations combined with auxin on bud breaking and shoot induction in *C. spinosa* var. *aegyptia*.

To stimulate the emergence of buds and growth of shoots, stem nodal segments of *C. spinosa* var. *aegyptia* were sterilized and cultivated on MS medium supplemented with different concentrations of cytokinins [6-benzyl adenine (BA), kinetin (Kin), N6-(2-isopentenyl) adenine (2iP), or thidiazuron (TDZ)] at 0.5, 1.0, or 3.0 mg/l combined with 0.5 mg/l α -naphthalene acetic acid (NAA). Murashige and Skoog (MS) medium without any plant growth regulators served as a control (Table 5). The cultures were kept at a temperature of 26 ± 2 °C under cool white fluorescent light with a photoperiod of 16/8 h light/darkness at an intensity of 2500–3000 lx. After eight weeks of cultivation, the percentage of growth induction, number of axillary shoots per explant, and length of axillary shoots were measured and recorded.

2.3.4 Effect of various combinations of cytokinins on the in vitro propagation of *C. spinosa* var. *aegyptia*.

To enhance the growth of *C. spinosa* var. *aegyptia*, the original shoots were transferred to a new medium (MS) that contained various combinations of cytokinins, BA, Kin, 2iP, and TDZ (as indicated in Table 6). The cultures were maintained under the same conditions as in the previous phase. After eight weeks of cultivation, the percentage of growth induction, the number and length of axillary shoots per explant were documented.

2.3.5 In vitro rooting of shootlets and hardening

The *in vitro* proliferating shootlets of *C. spinosa* var. *aegyptia* were cultured on a PGR-free MS medium for 4 weeks to remove any residual effects of PGRs that may reduce or exhibits an adverse effect on rooting. To initiate root growth, these shootlets were transferred to three different concentrations of MS basal medium (full, half, and one-fourth strength) supplemented with varying concentrations of α -Naphthalene acetic acid (NAA) alone or in combination with indole 3-butyric acid (IBA) (Table 7). The medium was solidified with 0.3% (w/v) phytagel and adjusted to a pH of 5.7. These cultures were incubated under the same conditions as the previous stages. The frequency of shootlets that developed roots and the number of roots per shootlet were documented four weeks after the transfer to the rooting media. The well-rooted plantlets were carefully washed to remove all medium residues and then transferred to pots containing a mixture of sand, soil, and peat moss (1:1:1) and covered with plastic bags in a greenhouse kept at a temperature of 28 ± 20 C and a humidity of 70–80%. The plants were regularly watered, and the covers were gradually removed after two weeks to allow for complete acclimatization. The percentage (%) of plants that survived after the acclimatization process was recorded.

2.3.6 Experimental design and statistical data analysis

The experiments were conducted five times with at least 40 replications and followed a completely randomized design. The collected data was analyzed using the analysis of variance (ANOVA) and Duncan's multiple range test [37], as modified by Snedecor and Cochran [38]. Results indicated that means labeled with the same letter are not significantly different at $P \leq 0.05$.

3 Results

3.1 Morphological identification

The plant specimen collected from its habitat, as depicted in Fig. 2, has been successfully identified at the species level with the help of the experts in the field as mentioned in the acknowledgment. The identification process was based solely on the floral structures and morphological characteristics, using the reference book Boulos [3]. Once identified, the plant specimen was assigned a voucher number for record-keeping purposes.

3.2 Molecular identification by DNA barcode analysis

In order to conserve *C. spinosa* var. *aegyptia*, the authentication of the plant was conducted at the molecular level. The molecular identification of *C. spinosa* var. *aegyptia* using DNA barcode analysis provides significant insights into the genetic makeup and phylogenetic relationships of this variety. By utilizing three barcode loci *trnH-psbA*, *matK* and *rbcl* and conducting BLAST searches, phylogenetic tree analyses and sequence comparisons, this study strengthens the understanding of the species' identity and evolutionary relationships. The sequences of the three barcodes were then submitted to the GenBank database with accession numbers OP995482, OP995481, and OP995480 for *trnH-psbA*, *matK*, and *rbcl*, respectively. The primers used in PCR amplification are listed in Table 1, and the resulting fragments had expected molecular weights of approximately 500, 900, and 600 for *trnH-psbA*, *matK*, and *rbcl*, respectively. Using BLAST matching and phylogenetic tree analysis, the plant species were identified as *C. spinosa* var. *aegyptia*. The alignments of the three sequences against GenBank accessions produced query coverage ranging from 95 to 100%, 100%, and 95% to 100% for *trnH-psbA*, *matK* and *rbcl*, respectively with an error value of 0.0% (Tables 2, 3 and 4 and Figs. 3, 4 and 5).

The *trnH-psbA* sequence analysis using the BLAST tool in the gene bank showed that the closest species to *C. spinosa* var. *aegyptia* is *C. spinosa*, with a 100% similarity. This confirms the genetic closeness between the variety and the species it belongs to. Among the different varieties of *C. spinosa*, the variety *mucronifolia* demonstrated a high similarity, with percentages ranging from 97.59% to 99.78%, indicating strong genetic relatedness between the varieties (Table 2). The construction of a phylogenetic tree based on the *trnH-psbA* sequence further illustrated the evolutionary relationships among these varieties, offering visual evidence of how *C. spinosa* var. *aegyptia* aligns within the species complex.

Table 2 DNA barcode of *trnH-psbA* downloaded from GenBank database including plant species with similarity percentage of more than 97.58% with *C. spinosa* var. *aegyptia*

Plant species	Accession no	E value	Query coverage (%)	Similarity (%)
<i>C. spinosa</i>	MT041701.1	0.0	100	100
<i>C. spinosa</i> var. <i>mucronifolia</i>	LC424491.1	0.0	96	99.78
<i>C. spinosa</i> var. <i>mucronifolia</i>	LC424489.1	0.0	96	99.13
<i>C. spinosa</i> var. <i>mucronifolia</i>	LC424492.1	0.0	96	98.70
<i>C. spinosa</i> var. <i>mucronifolia</i>	LC424488.1	0.0	96	98.70
<i>C. spinosa</i> var. <i>parviflora</i>	LC490113.1	0.0	96	98.92
<i>C. spinosa</i> var. <i>mucronifolia</i>	LC424490.1	0.0	95	97.59

Table 3 DNA barcode of *matK* downloaded from GenBank database including plant species with similarity percentage of more than 99.33% with *C. spinosa* var. *aegyptia*

Plant species	Accession no	E value	Query coverage (%)	Similarity (%)
<i>C. spinosa</i>	EU371772.1	0.0	100	99.60
<i>C. spinosa</i>	OK172369.1	0.0	100	99.34
<i>C. spinosa</i>	MZ277789.1	0.0	100	99.34
<i>C. spinosa</i>	MZ277788.1	0.0	100	99.34
<i>C. spinosa</i>	MZ277787.1	0.0	100	99.34
<i>C. spinosa</i>	MZ277786.1	0.0	100	99.34

Table 4 DNA barcode of *rbcl* downloaded from GenBank database including plant species with similarity percentage of more than 99.47% with *C. spinosa* var. *aegyptia*

Plant species	Accession no	E value	Query coverage (%)	Similarity (%)
<i>C. spinosa</i>	MZ277799.1	0.0	95	100
<i>C. spinosa</i>	MZ277800.1	0.0	95	100
<i>C. spinosa</i>	KU739588.1	0.0	100	99.83
<i>C. spinosa</i>	MZ277798.1	0.0	96	99.82
<i>C. spinosa</i>	MZ277802.1	0.0	96	99.82
<i>C. spinosa</i>	MZ277801.1	0.0	97	99.82
<i>C. spinosa</i>	AY167985.1	0.0	100	99.65
<i>C. spinosa</i> var. <i>ovata</i>	KU739586.1	0.0	100	99.48

Fig. 3 Phylogenetic tree of *C. spinosa* var. *aegyptia* using the cpDNA marker; *trnH-psbA*, showing names and accession numbers of plant species

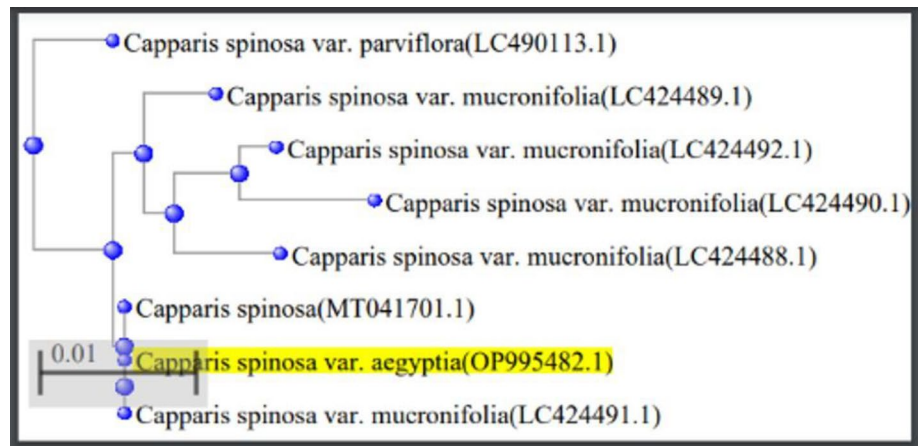


Fig. 4 Phylogenetic tree of *C. spinosa* var. *aegyptia* using the cpDNA marker; *matK*, showing names and accession numbers of plant species

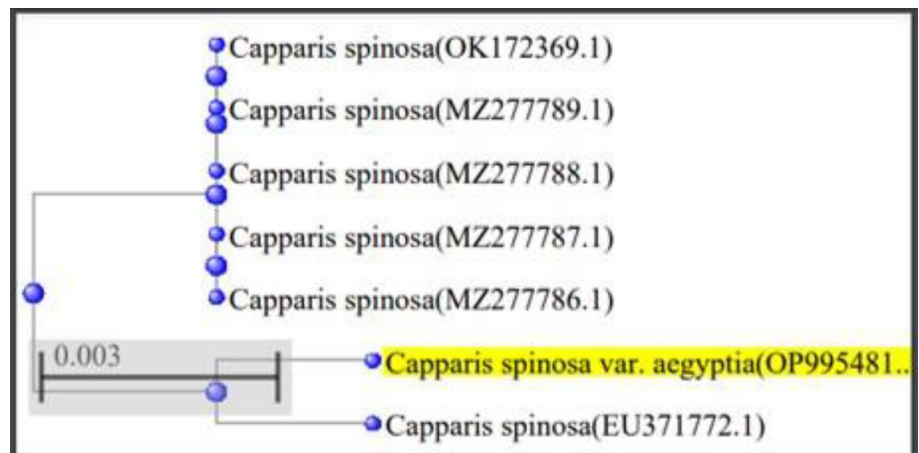
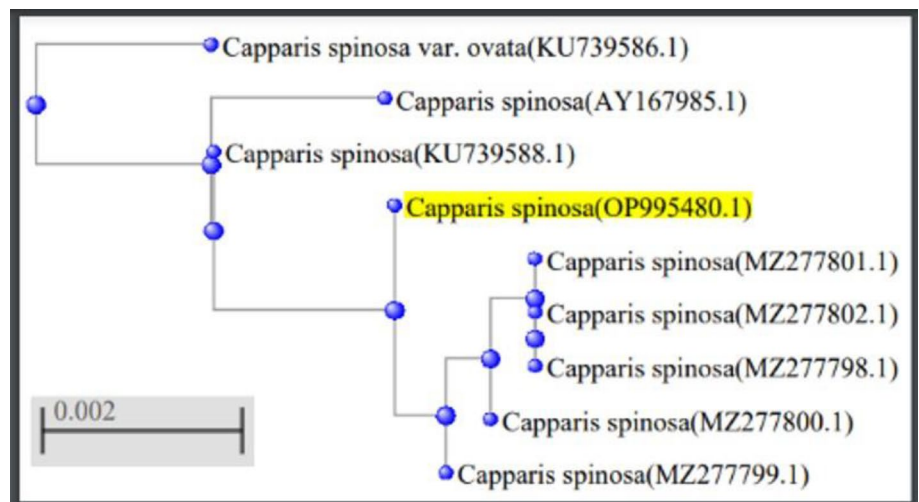


Fig. 5 Phylogenetic tree of *C. spinosa* var. *aegyptia* using the cpDNA marker; *rbcL*, showing names and accession numbers of plant species



This high degree of similarity suggests that *trnH-psbA* is a reliable marker for distinguishing *C. spinosa* var. *aegyptia* from closely related varieties, although it does not completely resolve the variety at a molecular level. The results underscore the challenge of identifying varieties within a species that share nearly identical DNA sequences (Fig. 3).

Similarly, the *matK* sequence analysis showed that the closest species to *C. spinosa* var. *aegyptia* is *C. spinosa*, with similarity percentages ranging from 99.34% to 99.60%. This high similarity reinforces the identification of the variety as part of the *C. spinosa* species complex (Table 3). The phylogenetic tree based on *matK* further supports this finding, as it

positions *C. spinosa* var. *aegyptia* alongside other varieties of *C. spinosa* (Fig. 4). The close genetic relationship evidenced by these findings highlights the usefulness of *matK* as a marker for species and variety identification, although the small genetic differences between varieties emphasize the need for additional markers to confirm the identity of varieties like *aegyptia*.

The *rbcl* sequence also demonstrated a high degree of similarity, with percentages ranging from 99.48% to 100% when compared to other species in the gene bank (Table 4). This suggests that *rbcl* is an effective marker for identifying *C. spinosa* and its varieties. The phylogenetic tree based on the *rbcl* sequence corroborates these results, showing a strong genetic connection between *C. spinosa* var. *aegyptia* and other species of the same genus (Fig. 5). This highlights the utility of *rbcl* in studying evolutionary relationships and suggests that it could be used as an additional tool for resolving genetic differences between closely related varieties.

3.3 In vitro propagation

3.3.1 Impact of various cytokinin types and concentrations combined with auxin on in vitro propagation of *C. spinosa* var. *aegyptia*

The recent study has revealed that stem nodal segments of *C. spinosa* var. *aegyptia* plants were survived at a rate of 60% using a 1.3% solution of sodium hypochlorite for a duration of 20 min. In this study, the impact of combining cytokinin and auxin on the growth and development of *C. spinosa* var. *aegyptia* was examined. Our results show that all cytokinins, including BA, Kin, 2iP, and TDZ, have a positive effect on the growth of shoots in MS medium supplemented with vitamins and sugar after 8 weeks of culture. However, the growth induction percentage, mean number of shoots, and mean length of shoots varied based on the type and concentration of cytokinin used. The growth induction percentage varied across different concentrations of cytokinins tested, ranging from 68 to 93% (Table 5).

Our findings indicate that 1.0 mg/l of BA + 0.5 mg/l NAA was the most effective combination for promoting shoot initiation and growth in *C. spinosa* var. *aegyptia*, resulting in a significantly higher percentage (93%) of shoots compared to other cytokinins at various concentrations. Additionally, this combination of BA and NAA also led to the highest mean number (4.3) and length of shoots (4.08 cm) (Fig. 6a). Furthermore, 1.0 mg/l of Kin, 2iP, or TDZ plus 0.5 mg/l NAA also significantly ($P \leq 0.05$) increased the growth induction percentage, the mean number and length of shoots compared to other treatments.

From Table 5 it was also observed that the lowest growth induction percentages, lowest mean number and length of shoots were observed in the medium supplemented with 0.5 mg/l NAA + 3.0 mg/l for each of TDZ, Kin, BA, and 2iP, respectively. Notably, explants cultured on MS medium without any plant growth regulators did not show any bud-breaking.

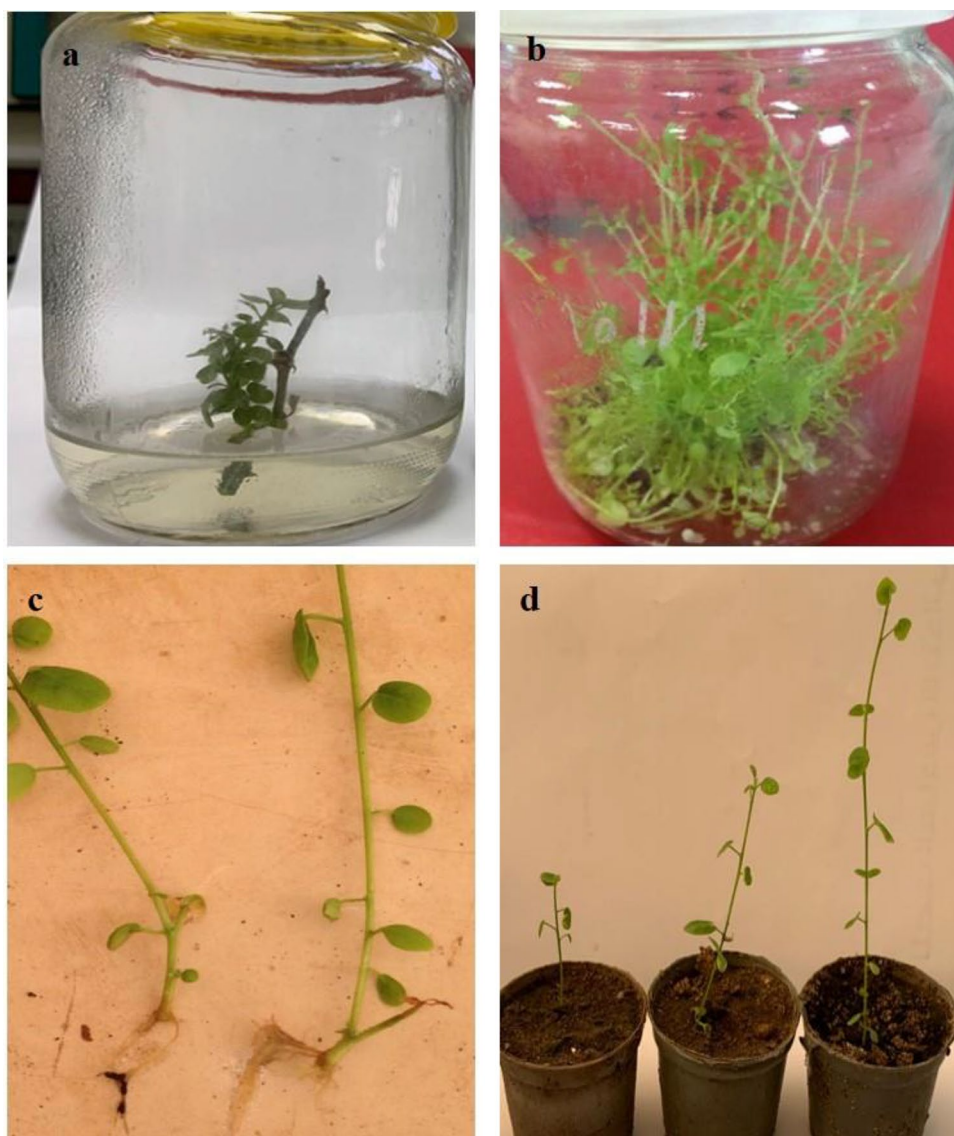
Table 5 Effect of different types of cytokinins in combination with 0.5 mg/l NAA on shoot induction from stem nodal segment explants of *C. spinosa* var. *aegyptia* cultured in full strength MS medium after 8 weeks of culture

Concentration (mg/l)				Growth induction % $\pm$ SD	Mean no. of shoots/explant $\pm$ SD	Mean length of shoot (cm) $\pm$ SD
BA	Kin	2-iP	TDZ			
0.0	0.0	0.0	0.0	00 $\pm$ 0.0 ^e	0.0 $\pm$ 0.00 ^d	0.00 $\pm$ 0.00 ^d
0.5	0.0	0.0	0.0	75 $\pm$ 0.5 ^c	2.3 $\pm$ 0.05 ^{bc}	2.16 $\pm$ 0.01 ^b
1.0	0.0	0.0	0.0	93 $\pm$ 0.6 ^a	4.3 $\pm$ 0.06 ^a	4.08 $\pm$ 0.01 ^a
3.0	0.0	0.0	0.0	68 $\pm$ 0.5 ^d	1.5 $\pm$ 0.05 ^c	1.07 $\pm$ 0.01 ^c
0.0	0.5	0.0	0.0	74 $\pm$ 0.5 ^c	2.0 $\pm$ 0.06 ^{bc}	3.66 $\pm$ 0.02 ^{ab}
0.0	1.0	0.0	0.0	77 $\pm$ 1.2 ^c	3.3 $\pm$ 0.05 ^{ab}	3.25 $\pm$ 0.02 ^{ab}
0.0	3.0	0.0	0.0	69 $\pm$ 0.2 ^d	1.3 $\pm$ 0.06 ^c	1.08 $\pm$ 0.01 ^c
0.0	0.0	0.5	0.0	75 $\pm$ 0.5 ^c	2.7 $\pm$ 0.05 ^b	2.08 $\pm$ 0.01 ^b
0.0	0.0	1.0	0.0	77 $\pm$ 0.5 ^c	3.0 $\pm$ 0.05 ^{ab}	3.41 $\pm$ 0.00 ^{ab}
0.0	0.0	3.0	0.0	69 $\pm$ 0.6 ^d	1.6 $\pm$ 0.05 ^c	1.41 $\pm$ 0.00 ^c
0.0	0.0	0.0	0.5	72 $\pm$ 0.6 ^c	2.7 $\pm$ 0.06 ^b	2.58 $\pm$ 0.01 ^b
0.0	0.0	0.0	1.0	82 $\pm$ 1.1 ^b	3.9 $\pm$ 0.05 ^a	4.25 $\pm$ 0.03 ^a
0.0	0.0	0.0	3.0	68 $\pm$ 0.5 ^d	1.0 $\pm$ 0.06 ^c	1.10 $\pm$ 0.04 ^c

*SD means standard deviation

In the same column, identical letters indicate that there is no statistically significant difference ($P \leq 0.05$)

Fig. 6 In vitro propagation of *C. spinosa* var. *aegyptia*; (a) bud breaking and shoot induction from stem nod segment explants, (b) multiplication of axillary shoots, (c) rooted plantlet, and (d) acclimatization of transplants in the greenhouse



Overall, our study suggests that 1.0 mg/l of BA + 0.5 mg/l NAA is the optimal concentration for promoting shoot initiation and growth in *C. spinosa* var. *aegyptia*. These findings could have significant implications for improving the cultivation and propagation of this species.

3.3.2 Impact of various combinations of cytokinins on the in vitro propagation of *C. spinosa* var. *aegyptia*.

During this experiment, the initial shoots of *C. spinosa* var. *aegyptia* were transferred to MS medium that was enriched with various combinations of cytokinins, including BA, Kin, 2iP, and TDZ (Table 6). Each combination produced a unique response in terms of shoot multiplication, with varying results observed. The combinations containing BA showed the highest growth induction percentages (95, 94 and 93% for BA with Kin, TDZ and 2iP respectively) compared to other combinations. However, the combination of 1.0 mg/l BA and 1.0 mg/l Kin in the MS medium resulted in the highest mean number of shoots (11.2) and the longest mean length of shoot (10.25 cm) (Fig. 6b). The second highest mean number of shoots (8.5) and second-mean length (9.0 cm) was obtained from cultures grown on medium containing 1.0 mg/l BA + 1.0 mg/l TDZ. In contrast, the lowest mean number of shoots (3.0) was recorded when shoots were cultured in the MS medium supplemented with 1.0 mg/l 2iP + 1.0 mg/l Kin. Significantly, explants grown on MS medium without the addition of any plant growth regulators did not exhibit any bud-breaking. These findings offer valuable insights into the most effective combinations of cytokinins for optimal shoot multiplication in *C. spinosa* var. *aegyptia*.

Table 6 Effect of different cytokinin combinations on shoot multiplication from stem nodal segment explants of *C. spinosa* var. *aegyptia* cultured in full strength MS medium after 8 weeks of culture

Concentration (mg/l)				Growth induction % $\pm$ SD	Mean number of shoots/explants $\pm$ SD	Mean length of shoots (cm) $\pm$ SD
BA	Kin	2-iP	TDZ			
0.0	0.0	0.0	0.0	00 $\pm$ 0.0 ^d	0.0 $\pm$ 0.00 ^e	0.00 $\pm$ 0.00 ^e
0.0	0.0	1.0	1.0	85 $\pm$ 0.6 ^b	6.5 $\pm$ 0.05 ^{ab}	2.0 $\pm$ 0.28 ^{cd}
1.0	0.0	0.0	1.0	94 $\pm$ 1.1 ^a	8.5 $\pm$ 0.50 ^b	9.0 $\pm$ 0.28 ^b
0.0	1.0	0.0	1.0	84 $\pm$ 0.5 ^b	4.5 $\pm$ 0.05 ^c	1.44 $\pm$ 0.01 ^d
1.0	1.0	0.0	0.0	95 $\pm$ 0.4 ^a	11.2 $\pm$ 0.50 ^a	10.25 $\pm$ 0.01 ^a
1.0	0.0	1.0	0.0	93 $\pm$ 0.3 ^a	5.0 $\pm$ 0.28 ^c	3.31 $\pm$ 0.03 ^c
0.0	1.0	1.0	0.0	79 $\pm$ 0.5 ^c	3.0 $\pm$ 0.05 ^d	2.0 $\pm$ 0.05 ^{cd}

*SD means standard deviation

In the same column, identical letters indicate that there is no statistically significant difference ($P \leq 0.05$)

3.3.3 In vitro rooting of the shoots and acclimatization

The study involved the excision and inoculation of healthy multiplied shoots of *C. spinosa* var. *aegyptia* to different strengths of MS medium fortified with various concentrations of NAA alone or in combination with 1.0 mg/l IBA. The aim was to induce root formation from the cut end of the shoots. The results showed that root formation was observed only in plantlets cultured in ¼ strength MS medium, and that the highest frequency of root induction (100%) was observed with 3.0 mg/l NAA treatments (Table 7 and Fig. 6c). The mean number of roots (4.67) and length of root were also highest in this concentration. The same concentration of NAA (3.0 mg/l) plus 1.0 mg/l IBA induced the second-best frequency of root induction (66.67%). However, ¼ strength MS medium containing 1.0 NAA or media containing 1.0 mg/l NAA plus 1.0 mg/l IBA did not induce any response. The control, which was ¼ strength MS medium without PGRs, also did not give any response.

Finally, the rooted shoots were successfully grown under greenhouse conditions and resulting in a survival rate of 95% (Fig. 6d).

4 Discussion

4.1 Molecular identification by DNA barcode analysis

DNA barcoding has emerged as a more precise alternative to traditional plant identification methods that rely on visible traits like seed or leaf shape. These traditional approaches can be hindered by environmental conditions or genetic variability, making it difficult to distinguish between closely related species [39–41]. By contrast, DNA barcoding uses standardized DNA sequences that are unique to each species, allowing for more accurate identification, even in incomplete or

Table 7 In vitro root initiation from *C. spinosa* var. *aegyptia* shoots under the influence of various concentrations and combinations of auxins in 1/4 strength MS medium

Concentration (mg/l)		Root induction (%) $\pm$ SD	Mean no of roots $\pm$ SD	Length of root(cm) $\pm$ SD
NAA	IBA			
0.0	0.0	00.00 $\pm$ 0.00 ^e	0.00 $\pm$ 0.00 ^d	0.00 $\pm$ 0.00 ^d
1.0	0.0	00.00 $\pm$ 0.00 ^e	0.00 $\pm$ 0.00 ^d	0.00 $\pm$ 0.00 ^d
2.0	0.0	22.00 $\pm$ 0.50 ^d	1.00 $\pm$ 0.06 ^c	0.50 $\pm$ 0.06 ^c
3.0	0.0	100.0 $\pm$ 0.00 ^a	4.67 $\pm$ 0.01 ^a	4.83 $\pm$ 0.01 ^a
1.0	1.0	00.00 $\pm$ 0.00 ^e	0.00 $\pm$ 0.00 ^d	0.00 $\pm$ 0.00 ^d
2.0	1.0	33.33 $\pm$ 0.02 ^c	2.00 $\pm$ 0.06 ^{bc}	1.25 $\pm$ 0.01 ^b
3.0	1.0	66.67 $\pm$ 0.02 ^b	3.67 $\pm$ 0.02 ^a	6.67 $\pm$ 0.01 ^a

*SD means standard deviation

In the same column, identical letters indicate that there is no statistically significant difference ($P \leq 0.05$)

damaged samples, and resolving distinctions between morphologically similar species [42]. This method also contributes to a global database, enabling consistent identification across different regions and conditions [43].

In this study, BLAST matching and phylogenetic tree analysis using three DNA markers; *trnH-psbA*, *matK*, and *rbcl* confirmed that *C. spinosa* var. *aegyptia* is genetically very close to *C. spinosa* and its varieties. It has been reported that species-level identification is considered successful when the similarity score exceeds 95%, while for genus-level identification, a score above 95% is required to include a single genus [44]. Here, the 100% similarity with *C. spinosa* and the high similarity with other varieties reaffirm that *C. spinosa* var. *aegyptia* belongs to the *C. spinosa* species complex. Due to the advocacy of the Consortium for the Barcode of Life (CBOL) plant working group, the *matK* and *rbcl* barcode was selected as they are considered a core barcode for plant species and *trnH-psbA* was chosen as a supplement barcode. Phylogenetic relationship studies are most effective when combining different barcodes to evaluate the evolutionary relationships between and within species [44]. Phylogenetic tree analyses using the three markers supported the inclusion of *C. spinosa* var. *aegyptia* within the *C. spinosa* species, showing its closest genetic relationship to the varieties *mucronifolia* and *ovata*. These findings highlight the effectiveness of DNA barcoding in identifying and conserving *C. spinosa* var. *aegyptia*, with significant implications in fields such as botany, medicine, and ecology. The molecular data presented here contribute valuable insights into the genetic makeup of the plant and serve as a reference for future research. Nonetheless, further studies using additional markers or techniques may be necessary to fully differentiate the varieties within *C. spinosa*.

4.2 In vitro propagation of *C. spinosa* var. *aegyptia*

Cytokinins promote shoot growth by driving cell division and balancing auxins. A high cytokinin-to-auxin ratio boosts shoot formation, optimizing conditions for multiple shoots per explant [45–47]. Stem nodal segments of *C. spinosa* var. *aegyptia* was successfully established in vitro on MS medium supplemented with 1.0 mg/l of BA + 0.5 mg/l NAA. BA proved to be the most effective cytokinin for promoting shoot proliferation, outperforming other cytokinins such as TDZ, Kin, and 2iP. The superior performance of BA may be attributed to its low toxicity, high activity, and stability, which result from the presence of natural ribosides and nucleotides, making it more effective than other cytokinins [48, 49]. The addition of 0.5 mg/l NAA supports shoot development by balancing cytokinin and auxin activity, promoting shoot formation without excessive root growth. The combination of BA (1.0 mg/l) with NAA participates in the shoot induction process in plants. This is supported by El-Mekawy et al. [13] who found that BA together with the NAA was effective in the micropropagation of *C. spinosa* L. Also, it has been reported by Carra et al. [27] and Attia et al. [29] that cytokinin with a small concentration of auxin are required in combination in tissue culture media and are the best for the initial shoot induction. BA's superior effectiveness in promoting shoot proliferation over other cytokinins has been well established in various studies, including *Pimpinella anisum* [50], *Bacopa monnieri* [51], *mount Parnitha* [52], *Plectranthus amboinicus* [53], and *Uapaca kirkiana* [54].

The optimal concentration of BA (1.0 mg/l) in combination with NAA reflects the synergy between cytokinin and auxin levels, where a balanced ratio is critical for effective shoot induction [55].

In this study, High cytokinin concentrations (above 3.0 mg/l) inhibited shoot proliferation due to hormonal imbalances and stress, reducing viability and growth. This aligns with previous study in in vitro establishment in *C. spinosa* conducted by Attia et al. [29], who observed fewer shoots with high BA concentrations. Similar inhibitory effects were seen in peach rootstock [56], *Salvia tomentosa* [57], *Prunus africana* [58], and almond-peach hybrids [59]. Optimizing cytokinin levels is essential for effective shoot proliferation, requiring careful testing to avoid negative effects.

Combining cytokinins (1.0 mg/l BA and 1.0 mg/l Kin) enhanced shoot multiplication in *C. spinosa* var. *aegyptia*, achieving 95% growth induction and 11.2 shoots per explant. The synergistic effect of combining cytokinins likely stimulates broader cellular responses, promoting both cell division and differentiation more effectively than using them alone. Similar benefits of cytokinin combinations were reported in *Capparis leucophylla* by Hegazi et al. [60] and in other species like *Camellia sinensi* [61], *Chlorophytum borivilianum* [62], *Paulownia* [63], and *Mitragyna parvifolia* [64].

Auxins are crucial for root initiation, growth, and elongation [65, 66]. In this study, quarter-strength MS medium was supplemented with NAA and IBA to induce rooting in *C. spinosa* var. *aegyptia*. The optimal rooting condition was found with 3.0 mg/l NAA, resulting in 100% rooting and the highest number of roots per explant. The variation in root induction is likely due to concentration-dependent responses, as higher auxin levels can inhibit root growth [67, 68]. NAA and IBA vary in their effectiveness depending on the plant species being propagated. NAA, due to its simpler and more stable chemical structure, is generally more effective for rooting, as it promotes the formation of root primordia, which are easier to induce than the adventitious roots stimulated by IBA. Therefore, NAA is often preferred in in vitro propagation. Several factors affect rooting efficiency, including the type and concentration of auxins and the specific plant species or clone [69]. Previous studies on *C. spinosa* have explored different responses to plant growth regulators (PGRs). Attia

et al. [29] achieved 95% rooting success after a 4-h pulse treatment with 100 mg/l IAA in darkness. Elmaghrabi et al. [12] reported that 5.0 mg/l IAA was the most effective for rooting on MS medium. El-Mekawy et al. [13] obtained 85% rooting with half-strength MS medium supplemented with 1.5 mg/l IBA and 2 g/l activated charcoal, while Gianguzzi et al. [14] found that 67% rooting was achieved with a liquid medium containing 0.75 mg/l NAA and 0.25 mg/l IBA. This study, in comparison, provides a comprehensive assessment of auxin concentrations for rooting in *C. spinosa* var. *aegyptia* and highlights the variability in responses across different research efforts.

Successful acclimatization of the in vitro propagated plants is essential for their survival in the field. In our study, the in vitro propagated *C. spinosa* var. *aegyptia* plants had a high survival rate (95%) during acclimatization in the greenhouse, indicating their adaptability to environmental conditions outside of the laboratory.

5 Conclusion

Protection of threatened species is a key priority of the 2030 Agenda and the Sustainable Development Goals (SDGs) [22]. To safeguard rare and endangered species, prompt action is essential. Biotechnology tools such as DNA barcoding and in vitro propagation are vital for conserving species both within and beyond natural reserves. DNA barcoding, combined with traditional morphological classification, enhances accurate species identification and genetic analysis [70]. Ex situ conservation via in vitro propagation offers high yields for commercial use and genetic improvement. This study marks the first molecular identification of *C. spinosa* var. *aegyptia* using DNA barcoding, alongside the development of an efficient in vitro propagation protocol, crucial for the species' conservation and intellectual property protection.

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Author contributions H.E. G. put the plan of the in vitro propagation section, did the DNA barcoding and analysis, written the manuscript and revised it, and corresponding the publication. S.S. M. Did the in vitro propagation of the plant and did the analysis of data. All authors read and approved the final manuscript.

Data availability The authors declare that the data supporting the findings of this study are available within the paper. The publicly available web links for the three accession numbers are: <https://www.ncbi.nlm.nih.gov/search/all/?term=OP995482> <https://www.ncbi.nlm.nih.gov/search/all/?term=OP995481> <https://www.ncbi.nlm.nih.gov/search/all/?term=OP995480>.

Declarations

Ethics approval and consent to participate The collection of the wild plant material for this study adheres to the national guidelines and directives of the Egyptian Ministry of Environment. This was part of a collaborative effort between the Desert Research Center and the Egyptian Ministry of Environment to conduct a botanical survey, as well as the collection and conservation of plant genetic resources in the Saint Katherine Protectorate, with no need for further confirmation.

Competing interests The authors declare no competing interests.

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