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Research article

Propagation method and germination condition of an arid and semi-arid species: *Withania frutescens* (L.)

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Abstract

Withania frutescens (L.), a member of the Solanaceae family, is a species found in arid and semi-arid areas and is recognized for its use in traditional phytotherapy. This study aims to develop *in-situ* and *ex-situ* conservation methods for this species. Seeds and cuttings were collected from different individuals of *W. frutescens*. The seeds underwent chemical and mechanical tests to assess their viability. For propagation, a second method, cuttings, was used with the application of hormones to improve rooting. The average dimensions of the seeds were 3 to 5 mm long, 2 to 3 mm wide, and 1 to 1.5 mm thick. The control treatment (water) showed no germination, while the hydrogen peroxide (H_2O_2) and hot water treatments achieved germination rates of 16.34% and 12.69%, respectively. After 12 weeks, the number of leaves (Nfe) was 4.89±2.53 in the third week and 22.88±8.71 in the twelfth week. The number of flowers (NFL) in the last week was 4.64±1.68 and the number of nodes (ND) was 5.60±1.78. Growth monitoring of the cuttings revealed a root callus rate of 2.51±0.74% for cuttings not treated with IBA, compared with 23.45±5.09% for those treated with IBA. *W. frutescens* is an endangered species and requires both *in-situ* and *ex-situ* conservation.

Keywords: *Withania*; Multiplication; Cutting; *in-situ*; *ex-situ*; Germination

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1. Introduction

Biodiversity is crucial for sustainable development and human well-being, but its rapid decline is endangers life on Earth. In many countries, particularly developing ones, the growing demand for biological resources is jeopardising the sustainability of this diversity (Canadell and Noble, 2001; Rankou et al., 2015). The conservation of Moroccan flora is now an absolute priority, particularly for species with aromatic and medicinal properties. We need to undertake concerted efforts to preserve genes, species and ecosystems, using combined *in-situ* and *ex-situ* conservation approaches (Scheldeman and Zonneveld, 2010).

In-situ conservation measures, such as the creation of protected areas, are essential for preserving ecosystems and natural habitats, as well as for restoring populations of threatened species to their native environment. However, it is important to recognise that small populations are particularly vulnerable to inbreeding and genetic erosion, which can lead to reproductive and dispersal problems (Buza et al., 2000; Menges, 1991; Morgan, 1999).

Withania frutescens (L.) is a species of the Solanaceae family, frequently used by the indigenous population to combat bacterial infections, conjunctivitis, inflammation, tuberculosis, stress, bronchitis, anxiety, neurological disorders, ulcers, liver disease and Parkinson's disease (Bellakhdar Jamal, 1998). Published studies have reported certain pharmacological activities of *W. frutescens*, including anti-inflammatory, analgesic and healing activities (El Moussaoui et al., 2020, 2021; EL Moussaoui

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et al., 2019). In this context, we carried out propagation tests on this plant using cuttings and germination methods to determine the best way of conserving and preserving this species in the face of over-exploitation by the indigenous population.

2. Materials and Methods

2.1. Identification of the plant studied

The plant is harvested during the flowering period in order to facilitate its identification. The plant is identified by a botanist from the biology department, with a voucher number (BPRN69).

2.2. Techniques for propagating the plant under study

In the conservation strategy, propagation by seed is a priority to ensure the genetic diversity of species. However, vegetative propagation can ensure the survival of a species by creating genetically identical offspring. Unlike animals and humans, plants contain undifferentiated meristematic cells at the start of their development, which can then differentiate into the specialised cells needed to produce the many organs of a developing plant. Thus, in ideal circumstances, a fragment of shoot, a branch, a piece of root or a leaf can give rise to a new plant with the same genetic information as the parent plant (Dubiez et al., 2023).

2.2.1. Seed multiplication

2.2.1.1. Morphological characterisation of fruits and seeds

Colour and form were assessed visually for fruits that were scattered or retrieved from the ground. Digital callipers were used to measure the dimensions of the fruit and the seeds. On average, ten fruits and one hundred seeds were used to determine the dimensions of the fruit and the seed, respectively. The seeds were sliced longitudinally and the fruits were cut transversely so that the insides could be examined. A digital camera was used to capture pictures of the seeds and fruits.

2.2.1.2. Cleaning of collected seeds

Mature seeds were collected at the four sites studied from 5 to 10 plants in each zone. The seeds were cleaned by passing them through a sieve, first with a 0.4 cm mesh to retain large impurities, then with a 0.2 cm mesh to eliminate dust and small impurities. After sieving, the last impurities are removed manually with a spatula, followed by rinsing to remove the substances covering the seed.

2.2.1.3. Water content

Seed moisture content was determined by measuring the weights of fresh and oven-dried samples at 45°C for 2 to 5 days using a precision balance (Sanogo et al., 2006). The moisture content was determined using the following formula:

$$MC = [(DW-FW)/FW] \times 100 \quad (1)$$

With MC: moisture content; DW: dry weight; FW: fresh weight.

2.2.1.4. Seed viability test

The seed viability test was carried out at the time of collection and after two months' storage at 4°C. Firm, yellow seeds with no deformations were recorded as healthy.

2.2.1.5. Buoyancy test

The solidity of the seeds was tested using a method commonly used by horticulturists: floating the seeds in water. Seeds that sink are considered viable, while those that float to the surface are considered non-viable. To confirm viability, we scarified the seeds without damaging them and then soaked them in tap water for 24 hours. This step allowed for complete hydration of the tissues, facilitating the extraction of the seed coats (Jawhari et al., 2023).

2.2.1.6. Tetrazolium test

After being washed in distilled water, dehulled seeds are let to soak in a 0.5% solution of triphenyltetrazolium chloride (TTC) at a temperature of around 40°C for a whole day. The seeds were washed many times with distilled water after dyeing in order to eliminate any excess colour. Next, we sort the seeds into two groups based on how they stain. In contrast to their uncolored counterparts, fully coloured seeds have a better chance of survival (Ferradous et al., 2017).

2.2.1.7. Seed germination tests

Aiming to eliminate seed dormancy and facilitate embryo-environment exchanges-specifically, water absorption, different batches of seeds were pregerminated according to different treatments (Hoareau, 2012; Nivot, 2005). Each treatment had three replicates, and each batch had twenty-five seeds.

The various scarification methods are described as follows: chemical scarification involves the use of concentrated sulphuric acid (H_2SO_4), where the seeds are soaked for one minute before being carefully rinsed under running water to remove any acid residue (Niang-Diop et al., 2010). Treatment with hydrogen peroxide (H_2O_2) involves immersing the seeds in hot water (40 °C) for an hour, then in H_2O_2 diluted to 50% for an hour, followed by exposure to pure hydrogen peroxide for 20 minutes (Benamar, 2005). A 0.2% solution of potassium nitrate (KNO_3) is also used to moisten the germination substrate at the start of the test (Rao et al., 2006). In addition, thermal scarification is carried out by immersing the seeds in hot water at 80°C to remove the waxy cuticle, while cold scarification involves placing the seeds on sand in sealed polythene bags, then storing them at 4 °C for 15 days (Li et al., 1999).

Before each pre-germination treatment, the treated material is sterilised in an alcohol solution, the aim being to eliminate any micro-organisms potentially harmful to germination. The seeds were then placed in petri dishes on substrate (charcoal soil and sand) sterilised in an oven (100°C for 1 hour), and on petri dishes containing filter paper sprayed with an antifungal agent (Fluconazole 0.20%). The seeds were sprayed with distilled water (every two days) to keep the substrate moist. A seed is considered to have germinated as soon as the radicle emerges. Temperature and light conditions alternate between day and night, each lasting 12 hours. Temperatures vary between 20 and 30°C. To better understand the physiological significance of the germinative behaviour of *W. frutescens* seeds, the number of germinated seeds was counted daily (Bouslama et al., 2007). The germination rate (GR) was

calculated using the mathematical formula (Jawhari et al., 2023):

$$GR\ (\%) = (Ng/Ns)*100 \qquad (2)$$

Where GR: germination rate (%); Ng: number of seeds germinated during the test; Ns: total number of seeds sown at the start of the test.

2.2.2. Vegetative propagation by cuttings

Rooting stem cuttings is the most widespread method of vegetative propagation for woody plants (Gaskins, 2007). This approach can be useful for ecological restoration purposes and for understanding the physiological processes of cuttings. In addition to these standards, there are two groups for physiological characteristics that contribute to the difficulty of seed germination:

- ✓ Internal reasons related to problems with biochemical processes for fruit and/or seed development that are not fully complete.
- ✓ External variables linked to the inability to control fully viable seed germination processes, such as the difficulty of obtaining seeds, a lack of biological understanding of how to germinate seeds, and the presence of agents that feed on seeds.

2.2.2.1. Collecting and handling cuttings

To provide a diverse genetic base, for example when restoring an ecosystem, it is preferable to take cuttings from a group of at least 10 widely spaced mother plants in the stand, and ideally from a range of 10 to 25 mother plants at each station. To preserve the totipotency of the cuttings, they were collected first thing in the morning, and the bag was watered every 60 minutes during collection and over long distances.

Two to three leaves were retained at each cutting to ensure that metabolic processes were not interrupted. The epidermis and central cylinder were treated carefully to avoid scarring. To avoid decomposition of the internode, the cuts were terminated at the base of a node. The fact that the top half of the cut had to be cut at an angle meant that the metabolic components of the cut (which are necessary for its existence) were preserved as much as possible throughout the cut, and also prevented desiccation, which would kill the cuts (Husen and Pal, 2006; Palanisamy and Kumar, 1997; Tousignant, 1995).

2.2.2.2. Treatments and measurement parameters

Vegetative propagation based on the viability of cuttings depends on three factors: the recovery of injured tissue, the reactivation of dormant buds and the redevelopment of previously non-existent organs. Three variables were therefore maintained throughout the observations: the rate of root callus formation, the total number of buds and the total number of leaves produced.

– Substrate

A good substrate must meet requirements such as porosity, aeration and maximum water retention. In this recipe, we have combined sand, potting soil (bought from BRICOMA-Fez), and ordinary soil with a percentage of 50%, 25% and 25%, respectively. For this reason, it is best to avoid planting the cuttings in biologically active soil, as this could encourage the development of fungi and other infections that could eventually invade the cuttings' root system.

– Indole Butyric Acid

Indole Butyric Acid (IBA) was used as a cutting hormone to promote the rooting problem of *W. frutescens* cuttings. IBA was prepared at different concentrations of the order of 1/2, 1/4, 1/8, 1/16, 1/64, 1/128 from a stock solution of 10 mg/ml. Each cutting was immersed in the solution for one minute, and 1 ml of IBA solution was added to each hole, four hours after the substrate had been well watered. To avoid loss of the liquid components of the hormone, watering was delayed until 48 hours after transplanting.

– Transplanting and daily maintenance

One or two nodes were implanted in the substrate when the cuttings were transplanted. For maximum retention of contents, the cuttings were planted at an angle. The highest, notched part was inclined towards the light source. As the cuttings fight desiccation and transpiration as soon as they are detached from their mother plant, humidity and temperature are crucial to the survival of the cutting. The cuttings were carefully watered twice a day, in the morning and late afternoon, to maintain a constant level of humidity

2.3. Statistical analysis

The results were processed using the normality test (Shapiro-Wilk) and the homogeneity test (Levene's test). The results were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Multiplication by seed of the plant studied

The fruits of *W. frutescens* are berries measuring 7-10 mm in diameter, with a reddish exterior and a fleshy, yellowish, fragrant endocarp. Each fruit contains an average of 10 seeds. The water content of the seeds is approximately 12.48%, and they have a rigid brown seed coat. The average length, width, and thickness of the seeds are 3-5 mm, 2-3 mm, and 1-1.5 mm, respectively (Figure 1). Viability tests, including floatation and TTC, revealed that all seeds were viable both at harvest and after two months of storage at 4°C

At maturity, a *W. frutescens* seed typically weighs 21.48 milligrams. The embryo is encased in endosperm and has a spiral form. As shown in Figure 1, the seed's cotyledons are inwardly bent, while the hypocotyl and radicle are outwardly curved. The radicle's tip is surrounded by a layer of endosperm known as the endosperm cap or micropylar endosperm, and another layer known as the lateral endosperm. The internal structure of the *W. frutescens* seed closely resembles that of the tomato. The cells in the endosperm cap have a smaller average width and thickness of the cell wall compared to the cells in the lateral endosperm. These differences indicate the area where radicle development is predisposed to take place.

Evaluation of the germination of *W. frutescens* seeds by seven methods showed a significant difference in germination rates among the different treatments. The control (water) showed no germination (Figure 2), whereas the hydrogen peroxide (H_2O_2) and hot water treatments resulted in germination rates of 16.34% and 12.69%, respectively.

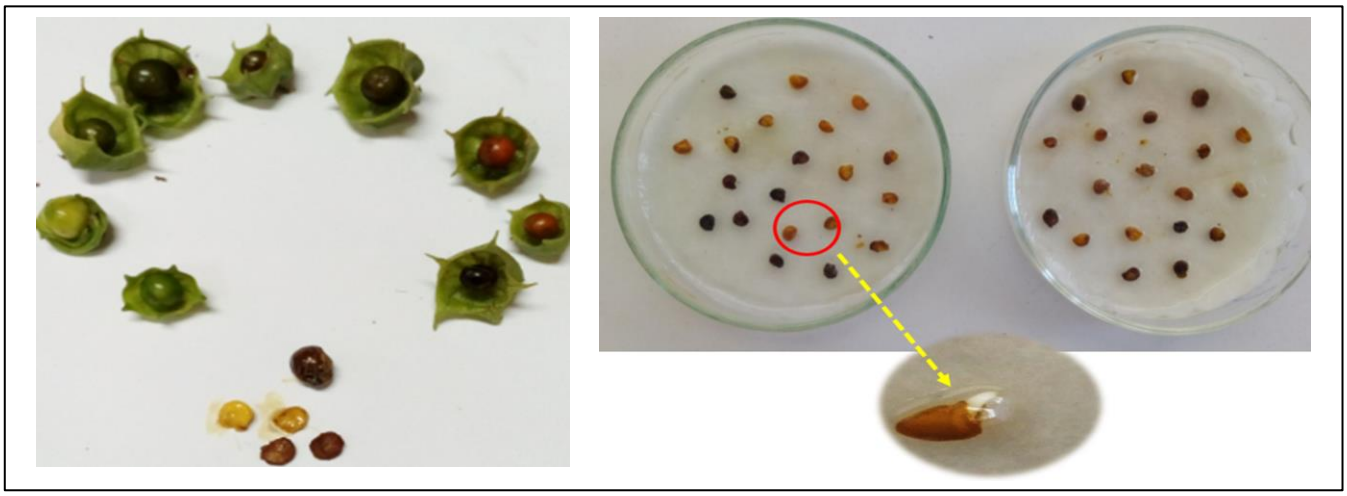

Figure 1: Fruits and seeds of *W. frutescens* at different stages of ripening (A) and seed germination (B)

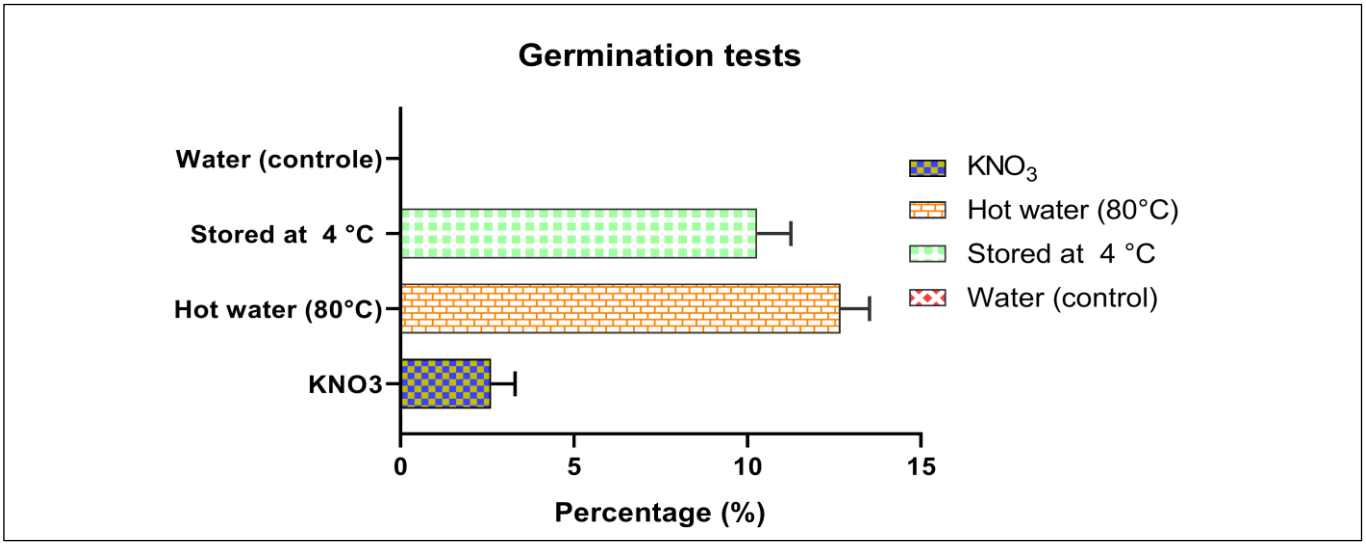

Figure 2: Germination results of *W. frutescens* seeds using various tests

Germination on filter paper was noticed, but no germination was observed in the sand used as substrate, which would mean that the plant needs light during the germination stage. Seedling development was characterised by elongation and the formation of secondary roots 3 days after radicle sprouting. Chlorophyll greening of the cotyledons began on day 4 and cotyledon opening 5 to 6 days after radicle sprouting. The cells of the endosperm cap in tomato, coffee, fenugreek, carob, and caraway seeds were found to be significantly different from the rest of the endosperm, which may play a role in radicle sprouting, according to previous research (Gong et al., 2005; Da Silva et al., 2004; Toorop et al., 2000).

The typical triphasic pattern of water uptake was observed in *W. frutescens* seeds over the 72-hour examination period. During the first phase, which lasts for approximately 12 hours after imbibition, the seeds rapidly absorb water and increase in fresh weight. This is followed by phase II, where the seeds' weight remains relatively stable until around 45 hours after imbibition. At this point, the fresh weight begins to rise again due to the root development of the fastest seeds in the population. For most seeds, however, phase III did not commence until after 50 hours. To allow the endosperm cap to burst and the radicle to emerge following testa rupture, the seeds needed to remain in a germination environment for an additional one or two days (Figure 3).

Additionally, the testa may play a role in providing the necessary puncturing force. Several Solanaceae species, such as tobacco and petunia, rupture the testa prior to radicle emergence as a result of embryo swelling and water absorption (Petruzzelli et al., 2003). Embryonic hypertrophy was observed in *W. frutescens* during phase III of germination. There was a substantial increase in the fresh weight of embryos derived from water-soaked seeds of *W. frutescens*. This observation suggests that the embryo underwent development during the process of imbibition and may potentially aid in the second phase of endosperm cap deterioration through an increase in pressure potential, known as turgidity.

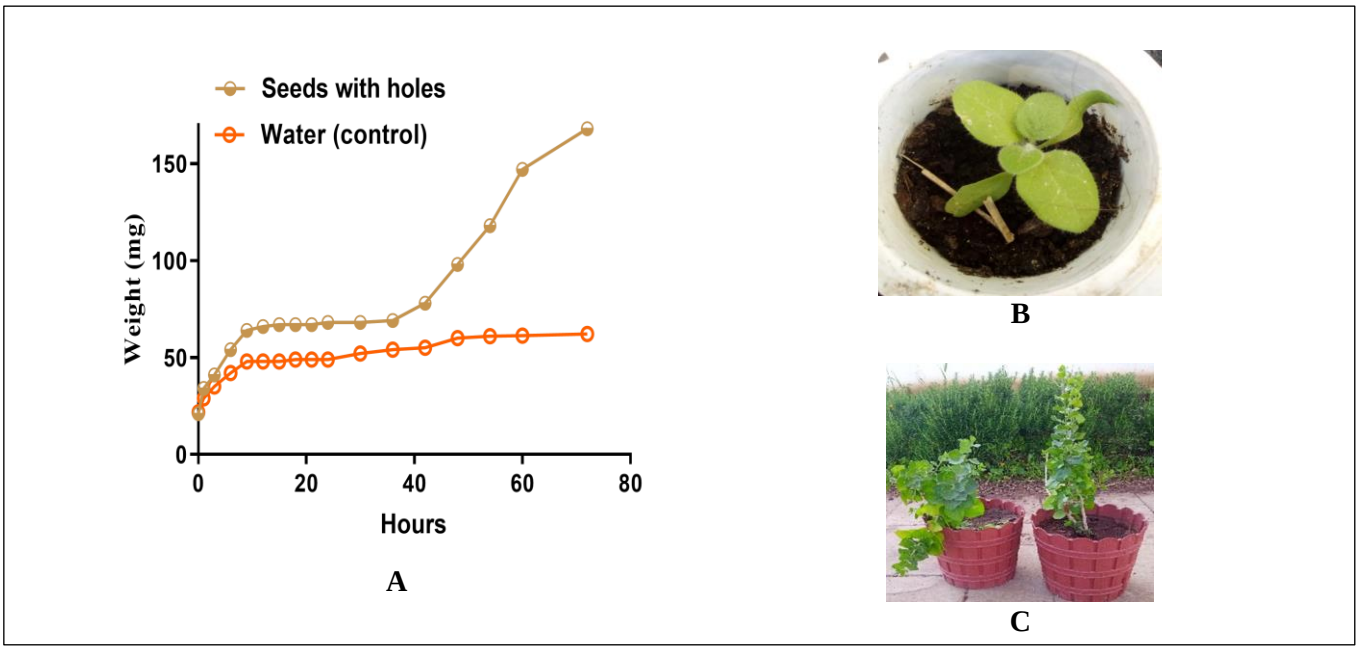

Figure 3: Change in seed weight as a function of time (A), plant development after germination (B and C)

Expansins are a class of proteins that facilitate cell division by compromising non-covalent bonds between cell wall polymers and interposing between cellulose and xyloglucan chains (Cosgrove, 1999, 2000). This enables expansion of the cell. The expression of the expansin gene, EXP8, is confined to the elongation zone of the radicle and is regulated by gibberellic acid and ABA in tomato seeds (Chen et al., 2001). To address the problem of root system rot in seedlings during transplanting, the soil was sterilized at a high temperature. After a few months, some of the transplanted plants were moved to the greenhouse at our Faculty of Science (Figure 3). It is important to note that *W. frutescens* seeds are fragile during both the germination period and transplanting, which could explain the low success rate of the germination propagation technique used. In this section, we also studied vegetative propagation of the species, focusing primarily on cuttings.

3.2. Vegetative propagation by cuttings

The cuttings technique provides a viable solution for ecological restoration, especially when faced with constraints such as the inability to naturally germinate seeds or a low germination rate, particularly when the species is restricted to a specific environment or is in a critical state.

The results of propagation by cuttings (Figure 4) show that there is no significant difference in the growth of the aerial part of the cuttings across the different types of soil used in this experiment, as measured by the number of leaves (Nfe), number of flowers (NFL), and number of nodes (ND). However, in woody trees, the concentration of mineral salts is preferable and plays an important role in rooting (Patil et al., 2007).

The crops remained green and healthy, also showing an indirect effect on the amount of regenerated shoots. And the development of new axillary shoots after two weeks of apical bud excision was observed in many seedlings as a consequence of the suppression of apical dominance and the induction of other meristematic dominances (Karalija et al., 2018).

Monitoring of growth parameters for 12 weeks shows that the *W. frutescens* cuttings began to grow from the second week, with changes in parameters observed until the last week. For example, the number of leaves (Nfe) increased from approximately 4.89±2.53 in the third week to about 22.88±8.71 in the twelfth week (Figure 4). The number of flowers (NFL) was around 4.64±1.68 in the last week, while the number of nodes (ND) was about 5.60±1.78 (Figure 4). During the monitoring period, the appearance rate of root callus in the cuttings was around 2.51±0.74% for those not treated with IBA. The development of a well-formed and functional root system is crucial for the absorption of water and minerals and for successful growth. Therefore, root branching is a critical step in overcoming poor root system development. The use of appropriate growing conditions, including ensuring contact between roots, air, and substrates in the medium, has been linked to improved root branching and enhanced seedling survival (Nowak and Shulaev, 2003). Nevertheless, treatment of cuttings with IBA showed a rate of appearance of root callus of the order of 23.45±5.09% as a peak in a concentration of 312.50 µg/mL of IBA. On the other hand, the high concentration (5 mg/mL) and the low concentration (78.10 µg/mL) showed a rate of around 0.0±0 and 8.91±1.42% (Figure 5), respectively. These results show that at low concentrations, root callus development can be observed, unlike at high concentrations where the rate is zero. The nature and concentration of auxins are specific to each species. In our study, root induction was only possible in a medium containing auxin. Other results indicate that IBA is the most suitable for root induction (Panwar et al., 2018; Teixeira da Silva et al., 2018), and is considered to be the most effective auxin in the rooting process (Nowak and Shulaev, 2003).

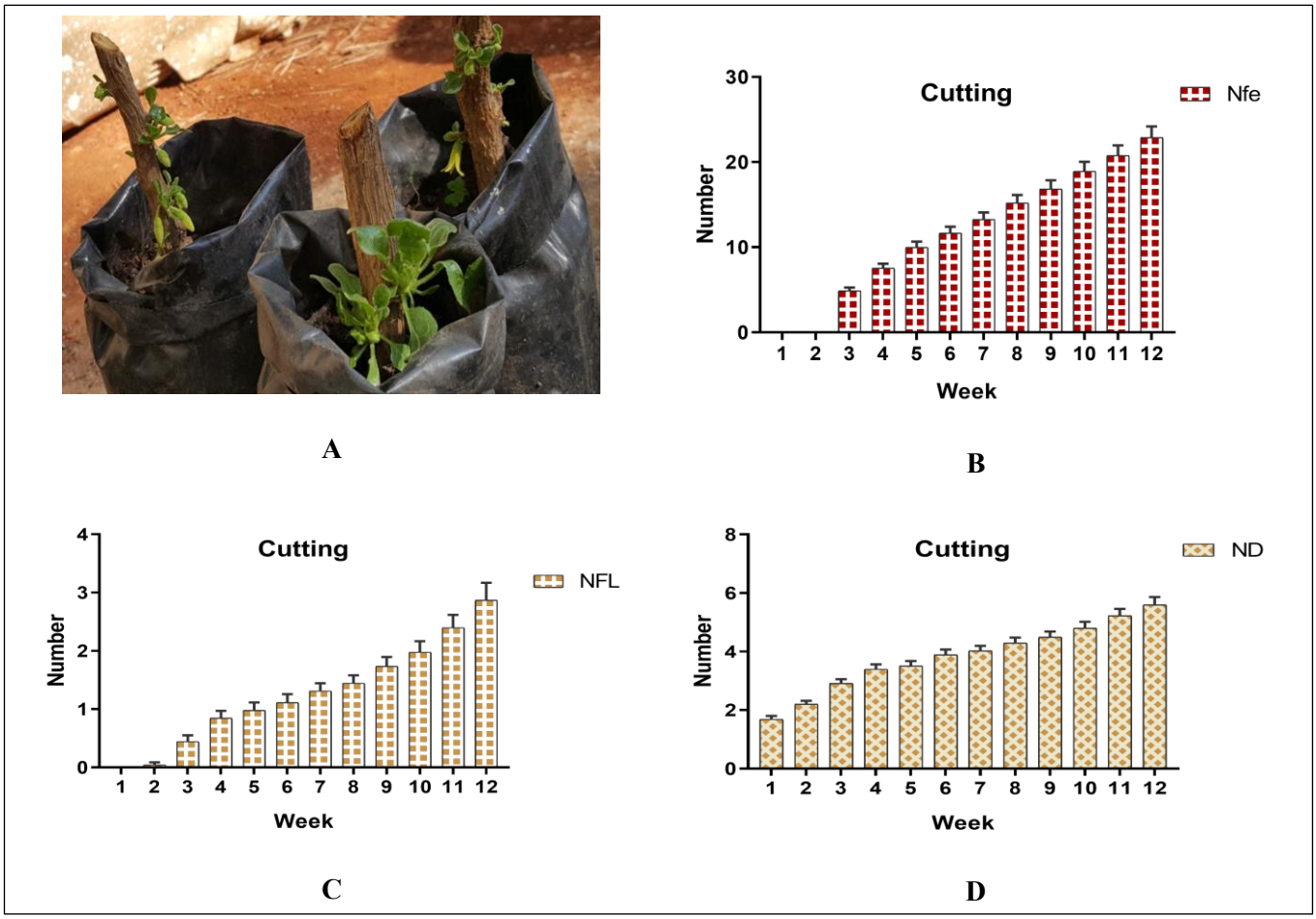

Figure 4. Cutting of *W. frutesecens* and growth monitoring by calculating the number of leaves (NFe) (B), the number of flowers (NFL) (C) and the number of nodes (ND) (D).

Auxin treatment is explained by the effect that the application of artificial auxins has on the existing balance between endogenous hormonal substances, resulting in improved rooting (Scholten et al., 1960). The auxins take effect without any risk of damaging the cuttings. And the rhizogenic capacity of the cutting depends on factors linked to the cutting itself and exogenous factors linked to the conditions to which the cutting is subjected (Fechtal et al., 2001).

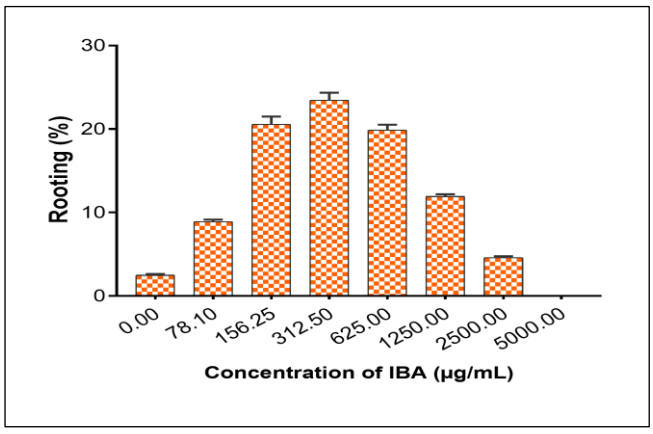

Figure 5. Rooting of *W. frutescens* cuttings under the influence of IBA

4. Conclusion

Withania frutescens (L.) is an endangered species in arid and semi-arid regions due to the difficulty of seed germination in the natural environment, attributed to the seed structure and resistance. The propagation methods used in this study demonstrated a low success rate in multiplying this medicinal plant. *W. frutescens* requires further propagation efforts at the laboratory level, such as through plant biotechnology. *In vitro* cultivation can be effective for conserving this species. Despite the low success rate of propagation by germination, multiple seedlings can be obtained from a mother plant using the micropropagation method.

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Conflicts of Interest: The authors declare no conflicts of interest.

Data availability statement: Data will be available upon request from the corresponding author.

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