An Efficient In vitro Rooting Protocol for Micropropagated Shoots of *Anagyris foetida L.*

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Abstract

Anagyris foetida L. is a woody leguminous species characterized by poor in vitro rooting, which represents a major obstacle to successful micropropagation. The present study aimed to develop an efficient rooting protocol for regenerated shoots following repeated failures of conventional rooting treatments. Initial experiments using MS and half-strength MS media supplemented with IAA or IBA, with or without activated charcoal, failed to induce root formation. However, transferring shoots to a hormone-free medium for four weeks prior to rooting successfully restored their rooting competence. The highest rooting response was achieved on half-strength MS medium supplemented with 2 mg L^{-1} IBA, resulting in 100% rooting after four weeks. Root initiation was observed during the first week of culture, with rooting percentages increasing progressively from 30%, 50%, and 70% in the first three weeks to 100% in the fourth week. Root number and root length also increased significantly throughout the culture period, reaching 3.50 roots per shoot and 3.45 cm, respectively. Rooted plantlets were successfully acclimatized and exhibited normal growth and establishment under ex vitro conditions without visible signs of transplanting stress. These findings demonstrate that hormone-free preconditioning followed by IBA treatment effectively overcomes rooting recalcitrance in *A. foetida* L. and provides a reliable protocol for large-scale propagation and conservation of this species.

Keywords: *Anagyris foetida*, micropropagation, adventitious rooting, IBA, half-strength MS medium, Acclimatization.

Introduction

Anagyris foetida L. is a perennial woody shrub belonging to the Fabaceae family and is naturally distributed throughout the Mediterranean region. The species has attracted considerable interest because of its ecological importance, drought tolerance, and potential use in reforestation and restoration programs in arid and semi-arid environments. Recent studies on A. foetida have mainly focused on seed dormancy, germination enhancement, and seedling establishment, whereas information regarding its *in vitro* propagation and rooting remains very limited [1]. Micropropagation is considered an effective tool for the rapid multiplication and conservation of valuable woody species. However, successful plant regeneration depends not only on shoot proliferation but also on the formation of a functional root system capable of supporting acclimatization and subsequent field establishment. Adventitious root induction is frequently regarded as the most critical stage of micropropagation, particularly in woody plants where poor rooting responses often limit the practical application of tissue culture protocols. The inability of regenerated shoots to form roots under in vitro conditions is commonly referred to as

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rooting recalcitrance. This phenomenon represents a major bottleneck in the propagation of many woody species and has recently received considerable attention because it restricts the large-scale production of elite genotypes. Recent advances have demonstrated that overcoming rooting recalcitrance requires not only exogenous auxin application but also the restoration of physiological competence for root initiation within regenerated tissues. Among the auxins used for root induction, indole-3-butyric acid (IBA) is considered the most effective and widely used rooting promoter because of its stability and its ability to stimulate root primordia formation in difficult-to-root species. Numerous studies have demonstrated the superiority of IBA over other auxins for adventitious root formation in woody plants. In addition to auxin supplementation, the mineral composition of the culture medium plays a decisive role in root induction. Several studies have reported that reducing the salt concentration of Murashige and Skoog (MS) medium improves rooting efficiency and root quality by decreasing osmotic stress and promoting root differentiation. In woody legumes, including Butea monosperma and Pterocarpus marsupium, half-strength MS medium supplemented with IBA produced superior rooting responses compared with full-strength MS medium. Despite the ecological and conservation importance of *A. foetida*, information regarding its *in vitro* rooting remains scarce. Available reports provide little information concerning rooting behavior, and no comprehensive protocol has been established to overcome rooting recalcitrance in regenerated shoots. Furthermore, previous rooting attempts in the present study using different concentrations of IAA and IBA, with or without activated charcoal, failed to induce root formation, indicating the existence of a pronounced rooting limitation. Studies on the micropropagation and rooting of *Anagyris foetida* remain extremely limited. [1,2] investigated the effect of selected growth regulators on the *in vitro* propagation of *A. foetida* and reported rooting responses under specific culture conditions. However, information regarding the restoration of rooting competence and the development of efficient rooting protocols for difficult-to-root shoots is still scarce. Although the study of [2] contributed to the development of a micropropagation system for A. foetida, rooting remained one of the major challenges, and the regenerated roots were limited in their development and quality. Therefore, further investigations were required to establish a reliable rooting protocol capable of producing vigorous root systems suitable for acclimatization and *ex vitro* establishment. Therefore, the present study aimed to develop an efficient protocol for overcoming in vitro rooting recalcitrance in *A. foetida* through hormone-free preconditioning followed by IBA treatment, and to evaluate the progressive weekly development of rooting, root growth, and subsequent acclimatization of regenerated plantlets.

Material and Methods

Plant Material

Shoots of *Anagyris foetida* L. were obtained from *in vitro* cultures maintained in the Plant Tissue Culture Laboratory, Department of Genetic Engineering, Biotechnology and Food Technology Research Center, Baghdad, Iraq. The shoots

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originated from a previously established micropropagation system and were maintained through regular subculturing on multiplication medium.

Rooting Experiments

To induce root formation, regenerated shoots measuring approximately 3–5 cm in length were excised and transferred to several rooting media were initially evaluated using full-strength MS and half-strength MS (½MS) medium [7], supplemented with different concentrations of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (1 and 2 mg L^{-1}). Activated charcoal (2 g L^{-1}) was also tested in selected treatments. All media contained 30 g L^{-1} sucrose and 7 g L^{-1} agar, and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Despite the use of different auxin types, concentrations, and medium strengths, no root formation was observed in any of the initial rooting treatments.

Hormone-Free Preconditioning and Root Induction

To overcome rooting recalcitrance, regenerated shoots were transferred to hormone-free MS and ½MS media containing 30 g L^{-1} sucrose and maintained for four weeks under standard culture conditions. Following this preconditioning phase, shoots were transferred to rooting media containing either 1 or 2 mg L^{-1} IBA. Cultures were maintained at 25 ± 2°C under a 16-h photoperiod provided by cool-white fluorescent lamps.

Evaluation of Rooting Response

Rooting response was evaluated weekly for four consecutive weeks. The following parameters were recorded:

Rooting percentage(%)

Number of roots per shoot

Root length (cm)

Rooting percentage was calculated according to the following equation:

Rooting percentage (%) = (Number of rooted shoots / Total number of cultured shoots) × 100

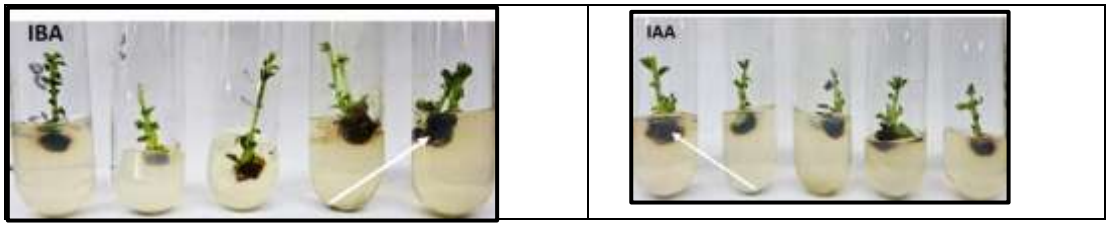
Acclimatization

Rooted plantlets obtained from the successful rooting treatment were carefully removed from the culture vessels and washed to remove adhering medium. Plantlets were transferred to pots containing a peat moss and sand mixture (1:1, v/v) and maintained under greenhouse conditions (Figure1 A). Relative humidity was gradually reduced during the acclimatization period. The acclimatized plants resumed normal vegetative growth and showed successful establishment under *ex vitro* conditions. (Figure 1B)

A



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B



Figure 1. *Ex vitro* acclimatization and establishment of rooted Anagyris foetida L. plantlets. (A) : Initial transfer of rooted plantlets to pots containing a peat moss:sand (1:1, v/v) substrate during the acclimatization phase. (B): Plantlets after two weeks of acclimatization, exhibiting successful establishment, active vegetative growth, and development of the apical meristem

Experimental Design and Statistical Analysis

The experiment was conducted using a completely randomized design (CRD). Each treatment was replicated three times, with a single culture vessel containing one shoot representing each replicate (experimental unit). Data for rooting percentage, number of roots, and root length were recorded weekly and subjected to analysis of variance (ANOVA) using GenStat software. Mean comparisons among weeks were performed using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ [3]

Results and Discussion

Rooting response of regenerated shoots

All conventional rooting treatments failed to induce root formation in regenerated shoots of Anagyris foetida. The use of MS and half-strength MS media supplemented with different concentrations of IAA and IBA, either alone or in combination with activated charcoal, did not result in root initiation. Instead, callus formation was frequently observed at the basal portion of the shoots, accompanied by tissue darkening (Figure 2). These observations suggest that the regenerated shoots exhibited a clear rooting recalcitrance, which could not be overcome by auxin application alone.

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Figure 2. Failure of adventitious root induction in micropropagated shoots of Anagyris foetida cultured on MS- and half-strength MS-based rooting media supplemented with IAA or IBA at concentrations of 1 and 2 mg L^{-1}, with or without activated charcoal. All treatments resulted in 0% rooting after four weeks of culture

Rooting recalcitrance is a common phenomenon in many woody legumes and has been attributed to physiological and hormonal imbalances that restrict root primordia initiation despite the presence of exogenous auxins. Similar difficulties have been reported in several woody members of the Fabaceae family, where successful shoot proliferation was not necessarily accompanied by root formation. Following a four-week culture period on hormone-free medium, rooting competence was restored and significant differences among rooting treatments became evident. Among all tested treatments, only half-strength MS medium supplemented with 2 mg L^{-1} IBA produced roots, yielding an average of 3.50 roots per shoot with a mean root length of 3.45 cm after four weeks of culture (Figure 3; Table 1, Table 2). In contrast, no roots were formed on the control treatment, 1 mg L^{-1} IBA treatments, or media containing activated charcoal.

Table 1. Effect of different culture media treatments on rooting percentage of Anagyris foetida L. shoots after four weeks of in vitro culture following the hormone-free preconditioning stage.

Treatments	Rooting percentage %
Control (0.0)	0.0 b
1/2 MS + 1 mg/l IBA	0.0 b
1/2 MS + 1 mg/l IBA + AC	0.0 b
1/2 MS + 2 mg/l IBA	100.0 a
1/2 MS + 2 mg/l IBA + AC	0.0 b

AC = Activated Charcoal (2 g L^{-1}). Means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

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Table 2. Effect of different culture media treatments on root number and root length of Anagyris foetida L. shoots after four weeks of ex vitro culture following the hormone-free preconditioning stage.

Treatments	Root numbers (root plant^{-1})	Root length (cm)
Control (0.0)	0.0 b	0.0 b
1/2 MS + 1 mg/l IBA	0.0 b	0.0 b
1/2 MS + 1 mg/l IBA + AC	0.0 b	0.0 b
1/2 MS + 2 mg/l IBA	3.50 a	3.45 a
1/2 MS + 2 mg/l IBA + AC	0.0 b	0.0 b

Means followed by the same letter within each parameter are not significantly different according to Duncan's Multiple Range Test (DMRT) at P ≤ 0.05.

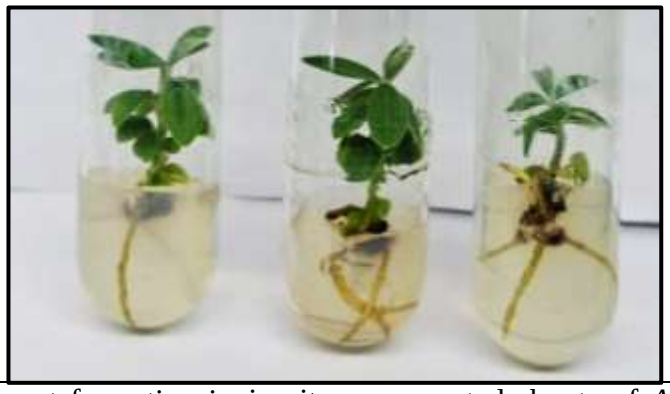


Figure 3. Adventitious root formation in *in vitro* regenerated shoots of *Anagyris foetida* L. cultured on rooting medium containing half-strength MS (½MS) supplemented with 2 mg L^{-1} IBA after four weeks of culture

The superiority of 2 mg L^{-1} IBA on half-strength MS medium may be attributed to the combined effect of reduced salt concentration and sufficient auxin availability for root primordia induction. Previous studies have demonstrated that half-strength MS medium often promotes rooting in woody species by reducing osmotic stress and favoring root differentiation. Likewise, IBA is widely recognized as the most effective auxin for adventitious root induction because of its stability and gradual conversion to biologically active auxin forms. Interestingly, activated charcoal completely inhibited rooting despite its positive effect on shoot appearance and vigor (Figure 4). This result may be explained by the strong adsorption capacity of activated charcoal, which can remove substantial amounts of IBA from the culture medium, thereby reducing auxin availability below the threshold required for root induction. Similar observations have been reported in

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several woody species where activated charcoal improved shoot quality but reduced rooting efficiency.

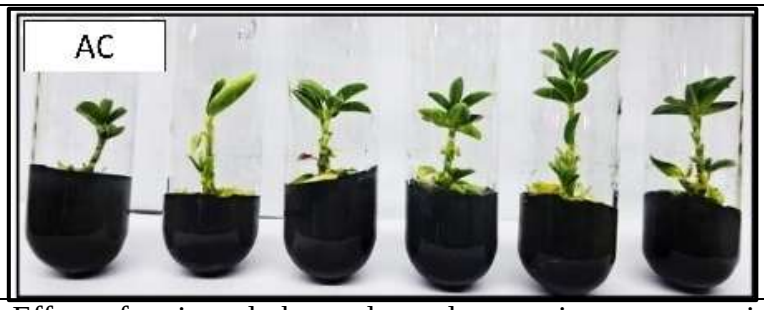


Figure 4. Effect of activated charcoal supplementation on vegetative growth and plant vigor of *in vitro*-derived *Anagyris foetida* L.shoots, inspite of no adventitious roots were formed, plantlets exhibited improved growth and overall morphological quality

Weekly progression of rooting development

Root formation progressed gradually throughout the culture period. The first visible roots appeared during the first week of culture, when rooting percentage reached 30%. Rooting percentage increased steadily to 50% and 70% during the second and third weeks, respectively, ultimately reaching 100% after four weeks (Figure 5; Table 3). A similar trend was observed for root number and root length. The average number of roots increased from only 0.30 roots per shoot during the first week to 3.50 roots per shoot by the fourth week. Likewise, root length increased continuously throughout the culture period, reaching 3.45 cm at the end of the experiment.

Table 3. Weekly progression of rooting percentage, root number, and root length in micropropagated shoots of Anagyris foetida L. cultured on MS medium supplemented with 2 mg L^{-1} IBA following the hormone-free preconditioning stage.

Weeks	Rooting percentage %	Root numbers (root $plant^{-1}$)	Root length (cm)
1	30 ± 15.3 c	0.30 ± 0.15 c	0.30 ± 0.15 c
2	50 ± 16.7 bc	0.80 ± 0.29 c	0.65 ± 0.22 c
3	70 ± 15.3 ab	1.50 ± 0.40 b	1.19 ± 0.30 b
4	100 ± 0.0 a	3.50 ± 0.40 a	3.45 ± 0.14 a

Values are presented as mean ± standard error (SE). Means followed by the same letter within a column are not significantly different according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

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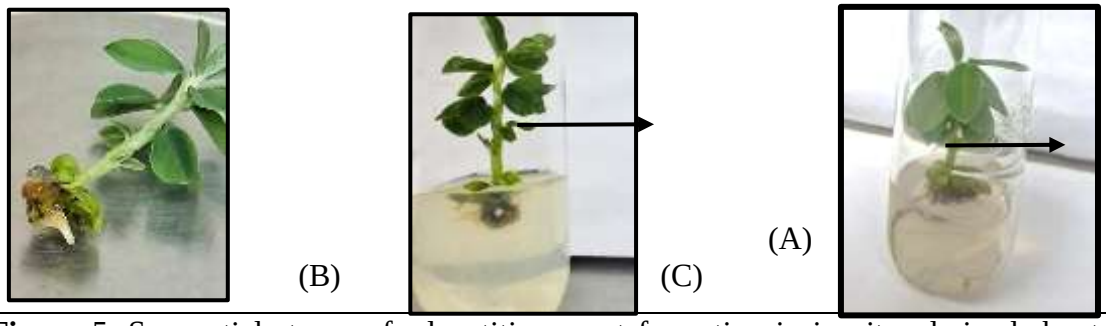


Figure 5. Sequential stages of adventitious root formation in in vitro-derived shoots of Anagyris foetida cultured on ½MS medium supplemented with 2 mg L^{-1} IBA: **(A)** root initiation during the first week, **(B)** root development during the second week, and **(C**) root elongation and establishment of a functional root system by the fourth week of culture

The gradual increase in rooting percentage, root number, and root length suggests that root induction and root elongation occurred in successive developmental stages rather than simultaneously. During the first week, the applied IBA likely stimulated the formation of root initials, whereas subsequent weeks were characterized by root emergence and elongation. This developmental pattern has been widely reported in adventitious rooting studies and reflects the sequential physiological events associated with root initiation, differentiation, and growth.

The progressive rooting response observed in the present study confirms that hormone-free preconditioning successfully restored the physiological competence of regenerated shoots. Exposure to cytokinin-free medium before root induction probably reduced residual cytokinin effects accumulated during shoot multiplication, thereby improving the endogenous auxin-to-cytokinin balance required for root initiation.

Acclimatization

Rooted plantlets obtained from the successful treatment were transferred to ex vitro conditions and acclimatized successfully. The plants resumed normal vegetative growth and showed no visible symptoms of transplant shock, wilting, or chlorosis. The successful acclimatization of rooted shoots indicates that the roots produced on half-strength MS medium supplemented with 2 mg L^{-1} IBA were functional and capable of supporting water and nutrient uptake under greenhouse conditions.

Discussion

The present study revealed that *in vitro* rooting of *Anagyris foetida* L. is a challenging stage in the micropropagation process. All conventional rooting treatments using different concentrations of IAA and IBA on MS and half-strength MS media

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failed to induce root formation. Similar difficulties have been reported in several woody species, where rooting is often the main obstacle limiting successful plant regeneration. [4,5] indicated that successful root induction depends not only on auxin application but also on the physiological competence of regenerated shoots to respond to rooting signals. A significant improvement in rooting was achieved after transferring shoots to hormone-free medium for four weeks before root induction. This preconditioning step appeared to restore rooting competence, likely through reducing the residual effects of cytokinins accumulated during the multiplication stage. [4] reported that the endogenous balance between auxins and cytokinins plays a decisive role in adventitious root formation, and disturbance of this balance may suppress root initiation even in the presence of exogenous auxins. Among all tested treatments, only ½MS medium supplemented with 2 mg L^{-1} IBA successfully induced rooting, producing 100% rooting after four weeks, with an average of 3.50 roots per shoot and a mean root length of 3.45 cm. The superiority of IBA agrees with previous studies that identified this auxin as one of the most effective growth regulators for adventitious root induction in woody species due to its stability and sustained physiological activity [5,6]. The beneficial effect of half-strength MS medium may be attributed to the reduced salt concentration, which promotes root differentiation and development compared with full-strength medium [7,8,9]. The gradual increase in rooting percentage from 30% in the first week to 100% in the fourth week, accompanied by continuous increases in root number and root length, reflects the sequential stages of adventitious root development, including root induction, emergence, and elongation. Similar rooting patterns have been reported in other difficult-to-root woody plants where root development requires an extended induction period before visible root emergence [5,9,10,11]. The successful acclimatization of rooted plantlets further confirmed the effectiveness of the developed protocol. The absence of visible transplant stress and the continued vegetative growth of acclimatized plants indicate that the roots formed on ½MS medium supplemented with 2 mg L^{-1} IBA were functional and capable of supporting plant establishment under *ex vitro* conditions. These findings demonstrate that hormone-free preconditioning followed by culture on ½MS medium containing 2 mg L^{-1} IBA provides an efficient approach for overcoming rooting recalcitrance in A. foetida and may contribute to future propagation and conservation programs for this species.

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