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Original Article

Callus and hairy Root Induction in the Medicinal Plant of *Withania coagulans* (Stocks) Dunal

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ABSTRACT

Callus induction from the leaf explants of *Withania coagulans* was assessed using the MS culture media containing co-application of BAP (0.1, 0.3, and 0.5 mg/L) and 2,4-D (0.1, 0.3, and 0.5 mg/L) based on a completely randomized design (CRD) with three replicates. Furthermore, the potential interaction effects of three different tissues (hypocotyl, stem, and leaf) of *W. coagulans* and two different *Agrobacterium rhizogenes* strains (R1000 and GM) were scrutinized for the hairy root induction using a factorial experiment based on CRD. For callus induction, among nine different hormonal treatments resulted from multiplying three concentrations of BAP and three concentrations of 2,4-D, one of which (i.e., "0.1 mg/L 2,4-D + 0.5 mg/L BAP") had the maximum amounts of fresh weight (3.024 g), dry weight (0.082 g), and callus volume (16.91 cm^3), as it was significantly different from the remaining eight hormonal treatments for the three traits (Duncan's test, $p< 0.05$). To make clear discrimination among nine different hormonal treatments and determine the best one(s) for callus induction in *W. coagulans*, a hierarchical cluster analysis (HCA) was also applied. All the treatments were placed in three main groups of I (six members), II (one member), and III (two members), of which the second cluster containing only one member (i.e., the hormonal treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP") had the highest quantities of the three aforesaid traits, could be accordingly proposed towards acquiring higher biomass. Regarding hairy root induction, no significant differences were observed either for interaction or for single effects ($p < 0.05$). However, among six different combinations (two strains and three tissue), the hairy root induction ratios ranged from 38.33% (GM/Hypocotyl) to 59.23% (for R1000/Leaf). Therefore, applying R1000 strain and leaf explant seems to be more effective for hairy root induction in *W. coagulans* compared to the other five combinations.

INTRODUCTION

Medicinal plants are still an important source in drug discovery, as more than 70-95% of people in developed countries use complementary or alternative medicines, many of which are related to herbal medicines [1]. The annual volume of global trade in herbal medicines is estimated to be 83 billion dollars, and is expected to increase significantly in the coming years [2]. However, the unsustainable and indiscriminate harvesting of medicinal plants from natural resources poses a major threat to these irreplaceable resources. Therefore, according to the recent advances in the field of biotechnology, the use of in vitro cultures such as callus culture and cell suspension followed by hairy root culture is recommended.

In Iran, a wide range of medicinal plants can be found, both in the wild and cultivated in fields, which mainly have food-medicinal properties. Among which, the genus *Withania*, the vernacular name "paneerbad" in Iran, is found in some parts of the country, particularly in the northeast and southeast. Due to its milk coagulation properties, the seeds/fruits powder of the plant is normally utilized by the indigenous people as sourdough to make local cheese [3]. For the genus *Withania* spp., a total of 61 species are known worldwide [4], two of which, *W. coagulans* and *W. somnifera* are very important in

terms of medicinal and economic properties, as cultivated widely in some countries such as India and Pakistan [5, 6]. The *W. coagulans* (Stocks) Dunal belongs to the *Solanaceae* family and is an endemic species in Iran (Baluchistan). Pharmacologically, different parts of the plant have antioxidant, antiinflammatory, antistress, antitumor, antimicrobial, and anticonvulsant effects [7, 8], which are mainly attributed to the presence of steroid lactones known as withanolides. The withanolides are chemical compounds that are naturally produced and have at least 300 different compounds. The most important types of withanolides in this plant species are withaferin A, withanolide A, and withanon [9].

Among different biotechnological approaches, hairy root culture has been recommended as a rapid and sustainable *in vitro* method for the production of various plant natural products applied in pharmaceuticals, food additives, and the cosmetic industry [10], followed by withanolides production [11-16], owing to having some advantages such as i) rapid growth in hormone-free medium; ii) ease of storage; and iii) capability to synthesize various chemical compounds [17]. In general, visible success towards hairy root induction could be affected by various issues such as genotype/species [18-20], type of plant tissue [16, 18, 20, 21], type of *Agrobacterium* strain [15, 16, 18, 20-25], and type of culture media composition [19, 23]. Furthermore, some chemicals like acetosyringone may also support hairy root induction [22, 23], albeit its concentration needs to be optimized from one study to another.

Regarding callus induction, several investigations have been accomplished in the genus *Withania*. For instance, callus induction was studied from the hypocotyl, root, and cotyledonary leaf segments of *W. somnifera* using the Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of 2,4-Dichlorophenoxyacetic (2,4-D) and kinetin [26]. The highest callus induction (100%) was acquired from the root and cotyledonary leaf explants grown on the MS medium supplemented with a combination of 2.0 mg/L 2,4-D and 0.2 mg/L kinetin [26]. Three different tissues of shoot tip, node, and leaf from *W. somnifera* were applied for callus induction using MS medium supplemented with of 2,4-D, Indole-3-butyric acid (IBA), kinetin, and benzyl aminopurine (BAP) ranging from 0.2 – 2.0 mg/L either alone or in combination [27]. Among three types of explants, leaf tissue was nominated as the superior explant followed by shoot tip and nodal explants based on callus formation [27]. Furthermore, the MS medium supplemented with 0.5 mg/L of 2,4-D and 0.2 mg/L kinetin gave the maximum callus induction (98%) [27]. The MS medium supplemented with combined or individual concentrations of thidiazuron (TDZ; 0.1, 0.5 and 1.0 mg/L), BAP (0.5 and 0.1 mg/L) and α-naphthalene acetic acid (NAA, 0.1, 0.5, 1.0 and 1.5 mg/L) was applied for callus induction from the intact leaves of *W. somnifera* [28]. The maximum quantities of callus formation (78%), callus fresh weight (3.5 g per explant), and callus dry weight (0.29 g per explant) were acquired on the MS medium containing 0.5 mg/L of each TDZ and NAA [28]. More recently, the MS culture media containing different concentrations of auxin hormones (0.0, 1.0, 1.5, 2.0 and 2.5 mg/L) such as 2,4-D, NAA, and IAA along with three different concentrations of BAP (including 0.0, 0.5 and 1.0 mg/L) have been used to study callus induction from the leaf and internodal parts of *W. coagulans* [29]. In general, the percentage of callus formation from the leaves (25-96%) was higher than from the internodal explants (23.23-85.4%). The highest percentage of callus was obtained from the leaf explants cultured in the MS medium enriched with "2.5 mg/L 2,4-D + 0.5 mg/L BAP" [29].

As *W. coagulans* is normally found in limited geographical areas and contains valuable medicinal compounds with high therapeutic properties, efforts to produce these compounds should be directed towards in vitro cultures. Therefore, in this study, the induction of hairy roots in *W. coagulans* was evaluated using two different strains of *Agrobacterium rhizogenes* as well as three different tissues (i.e., hypocotyl, stem, and leaf) of the plant. In addition, since different concentrations of auxin and cytokinin either alone or in combination result in various responses in terms of callus formation, the potential combination effects of two different hormones with three different concentrations were studied to induce callus in this medicinal plant species.

MATERIALS AND METHODS

PLANT MATERIALS

In this study, the seeds of *W. coagulans* were prepared from Barij Essential Pharmaceutical Company, Kashan, Iran. To produce sterile seedlings,

the seeds were placed in 70% alcohol for 40 seconds, washed thrice with distilled water, sterilized again using 5% sodium hypochlorite for 5 minutes, and finally washed thrice with distilled water. Lug jar bottles containing solid MS growth medium [30] supplemented with 30 g sucrose and 5.7 g/L agar (pH = 5.5-5.8) were prepared and several sterilized seeds were placed in each lug jar bottle, and the lid was sealed with parafilm. They were then transferred to the growth chamber with 16 hours of light and 8 hours of darkness (temperature 20-25 ºC). After obtaining strong 4-week seedlings, three explants of hypocotyl, stem, and leaf were applied for hairy root induction.

CALLUS AND HAIRY ROOT INDUCTION

To induce callus, leaf explants were prepared and cultivated in MS culture media including "*combined*" application of BAP (with three concentrations of 0.1, 0.3, and 0.5 mg/L) and 2,4-D (with three concentrations of 0.1, 0.3, and 0.5 mg/L). So, in total, we had nine different treatments resulted from multiplying three concentrations of BAP and three concentrations of 2,4-D. The explants were immediately transferred to the respective callus induction media and five explants were cultured in each petri dish. The petri dishes were kept inside the germinator at a temperature of 25 ± 2 °C under darkness. This test was conducted based on a completely randomized design (CRD) where each treatment was accompanied by three replications, and each replication contained a petri dish with five explants. After one month, the traits such as callus fresh weight (g), callus dry weight (g), and callus volume (cm^3) were recorded. To induce hairy roots, two different strains of *A. rhizogenes* known as R1000 and GM were used in this research. Both bacterial strains were isolated from the glycerol stock cultured in the solid LB medium containing the antibiotic rifampicin (50 mg/L) and kept in a refrigerator at 4 °C until use. The following three tissues of hypocotyl, stem, and leaf belonging to the 4-week sterilized seedlings (Fig. 1-a) were wounded by a scalpel blade dipped in bacteria (cultivated for 16-24 hours), while control explants were wounded only by a sterile scalpel blade. Inoculated explants were transferred into MS medium containing L-arginine and incubated for 3 days at ambient temperature of 25-26 °C. To eliminate additional bacteria, in the following, all the inoculated explants were washed thrice with sterilized distilled water containing 500 mg/L cefotaxime, transferred then into MS medium containing 3% sucrose, 0.75% agar, and 500 mg/L cefotaxime. This operation was repeated several times in the media containing cefotaxime (300 mg/L) to completely remove the bacteria. After the emergence of the roots, all the petri dishes were assessed based on hairy root induction (%).

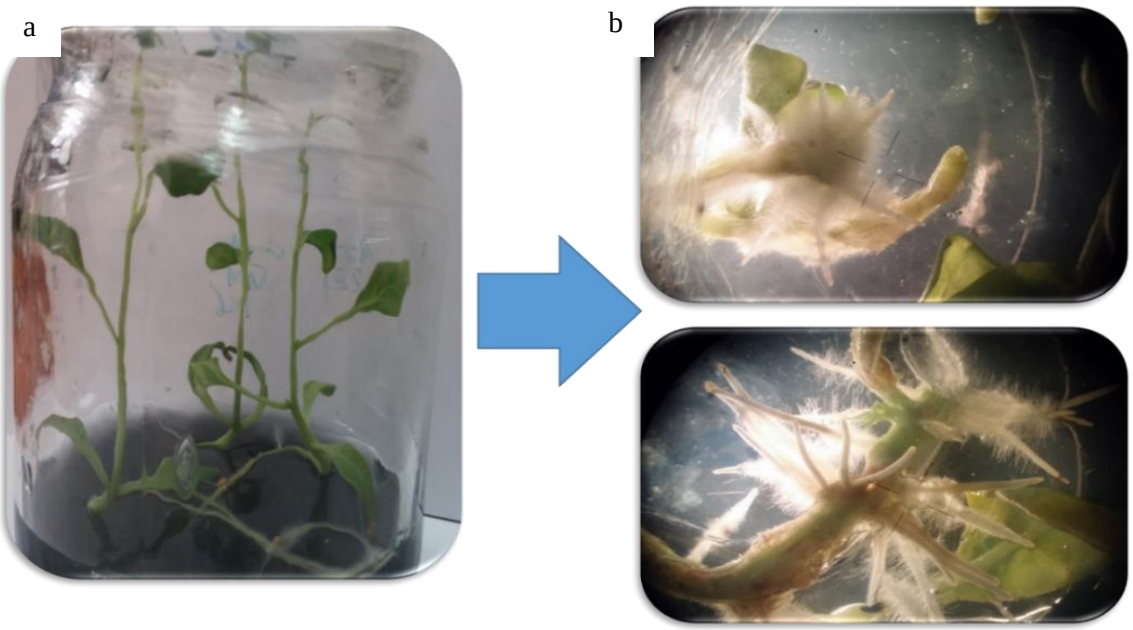

Fig. 1 Schematic representation of hairy roots in *W. coagulans* induced by two different strains of *A. rhizogenes* (i.e., R1000 and GM). a indicates 4-week sterilized seedling of *W. coagulans* grown in the lug jar bottle containing MS media, while b demonstrates induced hairy roots after the inoculation of explants with *A. rhizogenes*.

STATISTICAL ANALYSIS

For callus induction, the experimental design was based on a completely randomized design (CRD) with three replicates to determine potential differences among nine treatments (resulted from multiplying three concentrations of BAP and three concentrations of; totally, nine hormonal treatments) in terms of "*fresh weight*", "*dry weight*", and "*callus volume*". For hairy root induction, a factorial experiment with two independent factors (i.e., strain (S; two levels) and explant (E; three levels)) based on a CRD with three replicates (each replicate contained a petri dish with five explants over five consecutive weeks) was employed to determine differences in "*hairy root induction rate* (%)". ANOVA analyses were conducted via SPSS software Version 16, and the mean values were compared via Duncan's multiple range test at 5% probability level. To determine the best hormonal treatment(s) for callus induction, a hierarchical cluster analysis (HCA) combined with heatmap visualization was applied using ClustVis web-tool [31]. To achieve better understand among nine different hormonal treatments, pairwise correlation analysis was calculated via SPSS software Version 16.

RESULTS

HAIRY ROOT INDUCTION

In general, six different combinations resulted from multiplying two different bacterial strains and three different tissues of the plant were assessed in terms of hairy root induction in *W. coagulans*. Despite some fluctuations, both strains showed an ability to induce hairy roots in *W. coagulans* (Fig. 1-b). According to the ANOVA results, no significant differences were observed either for interaction or for single effects (Table 1; $p < 0.05$).

The quantities of hairy root induction for six different combinations of "GM/Hypocotyl", "GM/Leaf", "R1000/Hypocotyl", "R1000/Stem", "GM/Stem", and "R1000/Leaf" were ascendingly recorded as 38.33%, 41.11%, 50.00%, 50.95%, 57.94%, and 59.23%, respectively (Fig. 2). Even though the interaction effect was not statistically significant, as could be seen, the inoculation of leaf explant with R1000 strain seems to be more effective for hairy root induction in *W. coagulans* compared to the other five combinations. On the other hand, it appears that GM strain exhibited more affinity towards stem explant, as its hairy root induction value was still in high level of 57.89 %. Meanwhile, both R1000 and GM strains exhibited the least affinity when interacted with hypocotyl explant, as the lowest percentage of hairy root induction were observed after applying the same plant tissue.

Table 1 Analysis of variance to study the potential impacts of explants (E) and bacterial strains (S) in *W. coagulans* hairy roots based on "*induction rate* (%)"

Source of variation	df	Mean Square
E	2	121.4602 ^{ns}
S	1	259.996 ^{ns}
G*S	2	293.4937 ^{ns}
Error	12	151.0896

ns: No significant ($p< 0.05$)

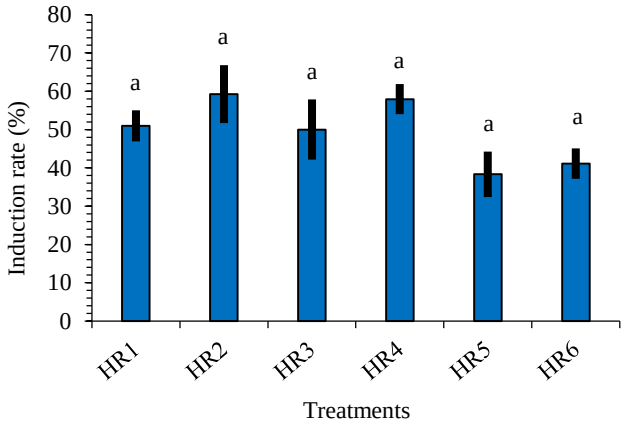

Fig. 2 Duncan's mean comparison test for the percentage of hairy roots induction roots in *W. coagulans* induced by the two different strains of *A. rhizogenes* (i.e., R1000 and GM). Similar letters above the bars indicate non-significant differences ($p > 0.05$). Data represent the mean of three replicates for each hairy root sample group ± SE. HR1-HR6 indicate six different hairy root treatments of "R1000/Stem", "R1000/Leaf", "R1000/Hypocotyl", "GM/Stem", "GM/Leaf", and "GM/Hypocotyl", respectively.

CALLUS INDUCTION

In general, after callus induction, nine different concentrations of both 2,4-D and BAP hormones exhibited significant differences on the fresh weight, dry weight and callus volume of *W. coagulans* ($p < 0.01$; Table 2).

To check which of the media was superior, a mean comparison was done based on Duncan's test. As shown in Fig. 3, for the callus fresh weight, all nine treatments were placed in five separate groups,

among which, the hormonal combination of "0.1 mg/L 2,4-D + 0.5 mg/L of BAP" (abbreviated as T3) was associated with the highest fresh weight of callus (3.024 g). The hormonal combination of "0.1 mg/L of 2,4-D + 0.1 mg/L of BAP" (abbreviated as T1; with an average fresh weight of 1.993 g) was ranked second. On the other hand, it seems that the culture media with the highest 2,4-D concentration (0.5 mg/L) have less effects on the growth rate and consequently callus weight, so that three different combinations of 2,4-D with the same concentration of 0.5 mg/L and average fresh weight of about 0.5 g were placed in the fifth group. This shows that the low concentration of 2,4-D and the high concentration of BAP can probably have the greatest effect on the production of callus with high level of fresh weight in *W. coagulans*.

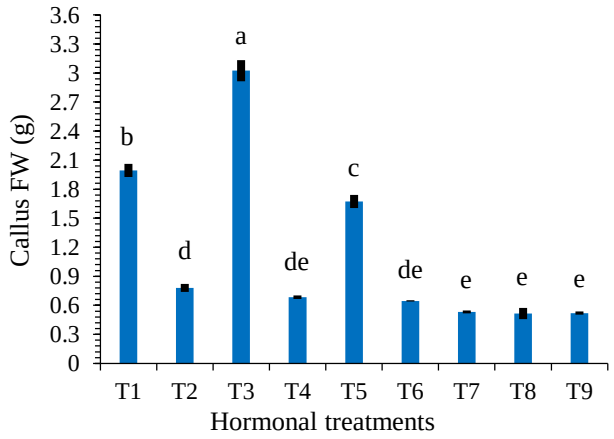

Fig. 3 Mean comparison of the exogenous application effects of nine different combinations of 2,4-D and BAP (each one with three various concentrations of 0.1, 0.3, and 0.5 mg/L) in the MS culture medium to induce callus in *W. coagulans* in terms of the criteria of "callus fresh weight (FW; g)". Among which, hormonal combination of T3 (i.e., "0.1 mg/L 2,4-D + 0.5 mg/L of BAP") was associated with the highest callus fresh weight of 3.024 g, while the remaining eight treatments induced the calli with lower fresh weights. Mean values indicated by different letters in a column are significantly different at $p < 0.05$, according to Duncan's multiple range test. Data represent the mean of three replicates for each hormonal combination ± SE. T1-T9 indicate nine different hormonal combinations/treatments of "0.1 mg/L 2,4-D + 0.1 mg/L BAP", "0.1 mg/L 2,4-D + 0.3 mg/L BAP", "0.1 mg/L 2,4-D + 0.5 mg/L BAP", "0.3 mg/L 2,4-D + 0.1 mg/L BAP", "0.3 mg/L 2,4-D + 0.3 mg/L BAP", "0.3 mg/L 2,4-D + 0.5 mg/L BAP', "0.5 mg/L 2,4-D + 0.1 mg/L BAP", "0.5 mg/L 2,4-D + 0.3 mg/L BAP", and "0.5 mg/L 2,4-D + 0.5 mg/L BAP", respectively

Although all nine treatments were placed in six separate groups according to their dry weight (Fig. 4), similar to the fresh weight, the hormonal combination of "0.1 mg/L 2,4-D + 0.5 mg/L BAP" (abbreviated as T3) resulted in the highest callus dry weight (0.082 g). The combination of "0.1 mg/L 2,4-D + 0.1 mg/L BAP" (abbreviated as T1) had the second highest average dry weight (0.053 g). On the other hand, all three media with the highest 2,4-D concentration of 0.5 mg/L (i.e., "0.5 mg/L 2,4-D + 0.1 mg/L BAP", "0.5 mg/L 2,4-D + 0.5 mg/L BAP", and "0.5 mg/L 2,4-D + 0.1 mg/L BAP"; abbreviated as T7, T8, and T9, respectively) had the lowest callus dry weight (0.014 g DW), and were placed in the sixth group.

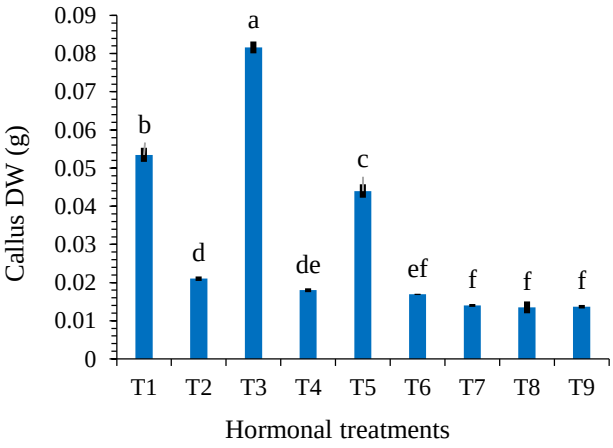

Fig. 4 Mean comparison of the exogenous application effects of nine different combinations of 2,4-D and BAP (each one with three various concentrations of 0.1, 0.3, and 0.5 mg/L) in the MS culture medium to induce callus in *W. coagulans* in terms of the criteria of "callus dry weight (DW; g)". Hormonal combination of T3 (i.e., "0.1 mg/L 2,4-D + 0.5 mg/L of BAP") was associated with the highest callus dry weight of 0.082 g, while the remaining eight treatments induced the calli with lower dry weights. Mean values indicated by different letters in a column are significantly different at $p < 0.05$, according to Duncan's multiple range test. Data represent the mean of three replicates for each hormonal combination ± SE. T1-T9 indicate nine different hormonal combinations/treatments of "0.1 mg/L 2,4-D + 0.1 mg/L BAP", "0.1 mg/L 2,4-D + 0.3 mg/L BAP", "0.1 mg/L 2,4-D + 0.5 mg/L BAP", "0.3 mg/L 2,4-D + 0.1 mg/L BAP", "0.3 mg/L 2,4-D + 0.3 mg/L BAP", "0.3 mg/L 2,4-D + 0.5 mg/L BAP', "0.5 mg/L 2,4-D + 0.1 mg/L BAP", "0.5 mg/L 2,4-D + 0.3 mg/L BAP", and "0.5 mg/L 2,4-D + 0.5 mg/L BAP", respectively.

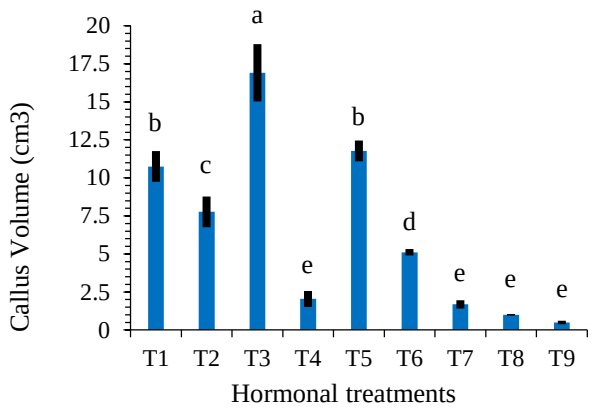

Fig. 5 Mean comparison of the exogenous application effects of nine different combinations of 2,4-D and BAP (each one with three various concentrations of 0.1, 0.3, and 0.5 mg/L) in the MS culture medium to induce callus in *W. coagulans* based on callus volume (cm^3). Hormonal combination of T3 (i.e., "0.1 mg/L 2,4-D + 0.5 mg/L of BAP" was associated with the highest callus volume of 16.91 cm^3, while the remaining eight treatments induced the calli with lower values of callus volume. Mean values indicated by different letters in a column are significantly different at $p < 0.05$, according to Duncan's multiple range test. Data represent the mean of three replicates for each hormonal combination ± SE. T1-T9 indicate nine different hormonal combinations/treatments of "0.1 mg/L 2,4-D + 0.1 mg/L BAP", "0.1 mg/L 2,4-D + 0.3 mg/L BAP", "0.1 mg/L 2,4-D + 0.5 mg/L BAP", "0.3 mg/L 2,4-D + 0.1 mg/L BAP", "0.3 mg/L 2,4-D + 0.3 mg/L BAP", "0.3 mg/L 2,4-D + 0.5 mg/L BAP', "0.5 mg/L 2,4-D + 0.1 mg/L BAP", "0.5 mg/L 2,4-D + 0.3 mg/L BAP", and "0.5 mg/L 2,4-D + 0.5 mg/L BAP", respectively.

CLUSTER ANALYSIS AND CORRELATION

Relatively similar results were obtained for the third trait (i.e. the volume of calli; Fig. 5). For example, all the nine studied treatments were divided into five separate groups, among which, the hormonal treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP" was the best hormonal combination with the maximum callus volume (16.91 cm^3) and the hormonal treatment of "0.1 mg/L 2,4-D (2,4-D) + 0.3 mg/L BAP" (T3; with an average volume of 11.78 cm^3) together with the hormonal combination of "0.1 mg/L 2,4-D + 0.1 mg/L BAP" (T1; with an average callus volume of 10.75 cm^3) were ranked second. On the other hand, and similar to the callus fresh weight, it seems that the culture media with the highest 2,4-D concentration (0.5 mg/L) had less effect on the growth rate and consequently callus volume, so that the three 2,4-D hormone combinations with the same concentration of 0.5 mg/L with an average callus volume lower than 1.68 cm^3 were placed in the fifth group.

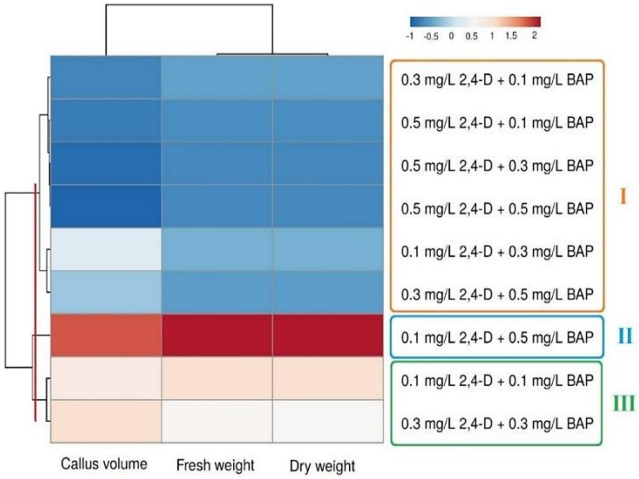

Fig. 6 Heat-map clustered for all the nine different hormonal treatments in callus induction of *W. coagulans* based on the quantities of three criteria of callus fresh weight (FW), callus dry weight (DW), and callus volume (V). According to the cutting line, all the nine treatments were placed in three separated groups of I (orange box containing six hormonal treatments), II (blue box containing just one hormonal treatment), and III (green box containing two hormonal treatments). Among which, the hormonal treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP" (cluster II; blue box) had the highest quantities of the three aforesaid traits (indicated by dark red color), could be accordingly proposed as the most effective hormonal combination for acquiring higher callus biomass. For each hormonal treatment, the mean values of three criteria were applied. To create hierarchical cluster analysis (HCA), both rows and columns were clustered using *Euclidean* distance and *Ward* linkage, respectively.

Table 2 Analysis of variance of the effect of nine different hormonal combinations on the callus induction in terms of callus fresh weight, callus dry weight, and callus volume in *W. coagulans*.

Source of variation	df	Mean Square (FW)	Mean Square (DW)	Mean Square (Callus volume)
Treatment	8	2.3577 ***	0.0017 ***	100.1686 ***
Error	18	0.0090	0.000004	2.1477

***: Significant at the 0.001 probability level

Table 3 Correlation coefficients among nine different hormonal treatments utilized in this study.

	0.1 mg/L 2,4-D + 0.1 mg/L BAP	0.1 mg/L 2,4-D + 0.3 mg/L BAP	0.1 mg/L 2,4-D + 0.5 mg/L BAP	0.3 mg/L 2,4-D + 0.1 mg/L BAP	0.3 mg/L 2,4-D + 0.3 mg/L BAP	0.3 mg/L 2,4-D + 0.5 mg/L BAP	0.5 mg/L 2,4-D + 0.1 mg/L BAP	0.5 mg/L 2,4-D + 0.3 mg/L BAP	0.5 mg/L 2,4-D + 0.5 mg/L BAP
0.1 mg/L 2,4-D + 0.1 mg/L BAP	1.000	0.997 ns	1.000 **	0.988 ns	0.999 *	0.998 *	0.991 ns	0.935 ns	0.613 ns
0.1 mg/L 2,4-D + 0.3 mg/L BAP	-	1.000	0.997 *	0.971 ns	0.999 *	1.000 *	0.976 ns	0.903 ns	0.546 ns
0.1 mg/L 2,4-D + 0.5 mg/L BAP	-	-	1.000	0.986 ns	0.999 *	0.999 *	0.99 ns	0.933 ns	0.608 ns
0.3 mg/L 2,4-D + 0.1 mg/L BAP	-	-	-	1.000	0.98 ns	0.977 ns	1.000 **	0.979 ns	0.730 ns
0.3 mg/L 2,4-D + 0.3 mg/L BAP	-	-	-	-	1.000	1.00*	0.984 ns	0.919 ns	0.579 ns
0.3 mg/L 2,4-D + 0.5 mg/L BAP	-	-	-	-	-	1.000	0.981 ns	0.913 ns	0.566 ns
0.5 mg/L 2,4-D + 0.1 mg/L BAP	-	-	-	-	-	-	1.000	0.975 ns	0.715 ns
0.5 mg/L 2,4-D + 0.3 mg/L BAP	-	-	-	-	-	-	-	1.000	0.853 ns
0.5 mg/L 2,4-D + 0.5 mg/L BAP	-	-	-	-	-	-	-	-	1.000

* and ** indicate significant at 0.05, 0.001 probability levels, respectively, while ns designates "No-significant".

AMONG HORMONAL TREATMENTS

To make clear distinction among nine different hormonal treatment(s) and determine the best hormonal treatment(s) for callus induction in *W. coagulans*, a hierarchical cluster analysis (HCA) combined with heatmap visualization was applied using ClustVis web-tool [31]. In general, according to the results of heatmap cluster analysis (Fig. 6), all the nine treatments were placed in three separate groups. The first group contained two hormonal treatments of "0.3 mg/L 2,4-D + 0.3 mg/L BAP" and "0.1 mg/L 2,4-D + 0.1 mg/L BAP" with moderate amounts of the three traits of fresh weight, callus dry weight, and callus volume. The second cluster was occupied only by one hormonal treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP", surprisingly with the highest quantities of the three aforesaid traits. The remaining six tretments formed the third group, with lower quantities of the traits.

To achieve better, understand among nine different hormonal treatments, pairwise correlation analysis was calculated. As indicated in Table 3, only 10 out of 36 correlation values calculated among nine different treatments were statistically significant. The treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP" (as the best hormonal treatment) correlated substantially with four treatments of "0.3 mg/L 2,4-D + 0.3 mg/L BAP", "0.3 mg/L 2,4-D + 0.5 mg/L BAP", "0.1 mg/L 2,4-D + 0.1 mg/L BAP", and "0.1 mg/L 2,4-D + 0.3 mg/L BAP". The second-best hormonal treatment of "0.1 mg/L 2,4-D + 0.1 mg/L BAP" exhibited positive significant correlation with the best hormonal treatment ("0.1 mg/L 2,4-D + 0.5 mg/L BAP") followed by "0.3 mg/L 2,4-D + 0.3 mg/L BAP", "0.3 mg/L 2,4-D + 0.5 mg/L BAP". The other significant and non-significant correlations are available in Table 3.

DISCUSSION

Plants are able to produce a wide range of secondary metabolites that are usually accumulated in small quantities, and usually some tissues of the plant may be dominant in producing higher levels of a specific compound compared to the other parts of the plant. Unfortunately, the isolation of such compounds from different genera of plants including *Withania* spp. is accompanied by harvesting from their habitat, and in some cases, overharvesting may result in narrowing their genetic pool. In this sense, alternative options like suspension culture, callus culture, adventitious roots, and hairy root have been recommended widely. Among which, the last one appears to be more preferable, mainly owing to its genetic stability, rapid growth, high growth ratio in hormone-free medium, sizable biomass, easy handling for large-scale biomass production in bio-reactors and elicitation [32, 33]. On the other hand, as mentioned above, hairy root induction is normally influenced by various issues such as genotype/species [18-20], type of plant tissue [16,

18, 20, 21], type of *Agrobacterium* strain [15, 16, 18, 20-23], and type of culture media composition [19, 23]. Furthermore, some chemicals like acetosyringone may also support hairy root induction [22, 23]. Therefore, for a given plant species like *W. coagulans*, possible interaction effects of a set of bacterial strains should be examined against different tissues of one or additional plant genotypes to acquire the optimal combination [23].

As shown in Fig. 2, the highest percentage of hairy root induction was observed for the leaf tissue inoculated with the R1000 strain (59.23%), and the two tissues of stem and hypocotyl had lower values (50.95% and 50.00%, respectively). For the GM strain, unlike R1000, the highest amount of hairy root induction was obtained for the stem explant (57.94%), and the leaf and hypocotyl tissues ranked in the next orders (41.11% and 38.33%, respectively). Therefore, it appears that GM and R1000 strains exhibited more affinity towards stem and leaf explants, respectively, concluded that hairy root induction in this plant species could be either "*tissue*" or "*strain*" dependent mechanism, as corroborated in the earlier investigations. The highest percentage of hairy root induction was obtained for the R1000 strain (50.6%), while both MTCC 2364 and MTCC 532 strains had lower values of 29.3% and 18.6%, respectively [15]. These results were in consistent with the findings of the current study, indicating that R1000 strain had a greater relative advantage for hairy root induction in *W. coagulans* compared to the GM strain. The successful Ri T-DNA transferring into the host cells is assumed as a key determinant towards the process of infection using *Agrobacterium* strains [15], leading to a more efficient induction of hairy root formation [22]. Based on our results as well as some earlier reports in different plant species like *W. somnifera* [15] and *Fagopyrum tataricum* Gaertn [34], this fundamental determinant of Ri plasmid must be greater in R1000 than the other strains [15], albeit in some cases other strains may have comparable and higher intrinsic efficacies than R1000 [24, 25]. On the other hand, it appears that GM strain exhibited more affinity towards stem explant, as its hairy root induction value was still in high level of 57.89 %. Meanwhile, both R1000 and GM strains exhibited the least affinity when interacted with hypocotyl explant, as the lowest percentage of hairy root induction were observed after applying the same plant tissue.

It has been shown that foliar explants from the plants grown in greenhouse (with a lifespan of 75 days) had a better response to induce hairy roots compared to the explants from in vitro culture [35]. By now, different strains of *A. rhizogenes* followed by various explants have been employed to assess their ability to induce hairy roots in *W. somnifera* [17]. However, with respect to the hairy root culture and callus induction in *W. coagulans*, few studies have been conducted so far. For example, in recent research conducted by Mishra *et al*. (2013), leaf explants were used to induce hairy roots by *A. tumefaciens* strain LBA4404 in *W. coagulans*. According to the results, the percentage of hairy root induction was reported as 80.05% [36]. In another study, Mirjalili *et al*. (2009) used leaf explants to induce hairy roots by *A. tumefaciens* strain C58C1 in *W. coagulans*, and the percentage of hairy root induction was recorded as 95% [37]. Of the hairy roots obtained, nearly 45% had a callus-like structure, while the remaining 55% had a normal appearance similar to hairy roots [37]. In another study, three *Agrobacterium* strains of R1000, MTCC 2364, and MTCC 532 were used to induce hairy roots in the leaf explants of *W. somnifera* [15]. Finally, in another study, three strains of *Agrobacterium* (MTCC 532, ATCC15834 and A4) were used to investigate their effects on hairy root induction from leaf explants and shoot tips of *W. somnifera* [16]. According to the results of the same research, the efficiency of hairy root induction by ATCC 15834 strain was approximately twice as high as that of the other two bacterial strains [16]. Furthermore, for the superior strain, the percentage of hairy root induction for leaf explants and shoot tips was reported to be 66.5% and 59.5%, respectively [16]. At the study of [11], transformation rate was as high as 90 % in the *W. somnifera* leaf explants infected with R1000 strain, followed by generation of 28.2 hairy roots per explant upon 12 days of culture. Considering the preference for opines, the *A. rhizogenes* strains are usually detached into five agropine, nopaline, mannopine, octopine, and cucumopine groups, of which agropine-type strains like R1000 and ATCC 15834 have been applied widely, actually owing to possessing high efficacy for hairy root induction [16, 17]. To induce hairy root culture in *Salvia virgata*

[38] and *Catharanthus roseus* [39], agropine-type strains (e.g., ATCC 15834, R1000, and A4) containing two T-DNA regions on their Ri plasmid (TL and TR) had more infection capability than mannopine-type strains like C58C1. Such different results could be due to different interactions that might be occurred between bacterial strains and different plant species from different genera.

Regarding callus induction, a number of investigations have been accomplished in different medicinal plant species [40] including the genus *Withania*. For instance, callus induction was studied from the hypocotyl, root, and cotyledonary leaf segments of *W. somnifera* using the MS medium supplemented with various concentrations and combinations of 2,4-D and kinetin [26]. The highest callus induction (100 %) was acquired from the root and cotyledonary leaf explants grown on the MS medium supplemented with a combination of 2.0 mg/L 2,4-D and 0.2 mg/L kinetin [26]. After one month, the highest ratios of fresh and dry weights (0.89 and 0.100 g, respectively) were acquired for the calli derived from cotyledonary leaf segments grown on the MS medium comprising 2.0 mg/L 2,4-D and 0.2 mg/L kinetin [26], a low fresh and dry weight compared to the current study (Fig. 3-4). In another study, three different tissues of shoot tip, node, and leaf explants from *W. somnifera* were applied for callus induction using MS medium supplemented with of 2,4-D, IBA, kinetin, and BAP ranging from 0.2 – 2.0 mg/L either alone or in combination. Among three types of explants, leaf tissue was nominated as the superior explant followed by shoot tip and nodal explants based on callus formation [27]. Furthermore, the MS medium supplemented with 0.5 mg/L of 2,4-D and 0.2 mg/L kinetin gave the maximum callus induction (98%) [27]. The MS medium supplemented with combined or individual concentrations of thidiazuron (TDZ; 0.1, 0.5 and 1.0 mg/L), BAP (0.5 and 0.1 mg/L) and α-naphthalene acetic acid (NAA, 0.1, 0.5, 1.0 and 1.5 mg/L) was applied for callus induction from the intact leaves of *W. somnifera* [28]. After five weeks, the maximum quantities of callus formation (78%), callus fresh weight (3.5 g per explant), and callus dry weight (0.29 g per explant) were acquired on the MS medium containing 0.5 mg/L of each TDZ and NAA [28], while the cytokinin BAP failed to induce callus (an inhibitory role), possibly due to its contribution to provoke ethylene biosynthesis [28]. Both callus fresh and dry weight were in the ranges of those reported in the current study (Fig. 3-4). More recently, MS culture media containing different concentrations of auxin hormones (0.0, 1.0, 1.5, 2.0 and 2.5 mg/L) such as 2,4-D, NAA, and IAA along with three different concentrations of BAP (0.0, 0.5 and 1.0 mg/L) have been used to study callus induction from the leaf and internodal explants of *W. coagulans* [29]. In general, the percentage of callus formation from leaves (25-96%) was higher than from internodal explants (23.23-85.4%). The highest percentage of callus was obtained from the leaf explants cultured in MS medium enriched with "2.5 mg/L 2,4-D + 0.5 mg/L BAP" [29]. In general, based on the results of this research, the hormonal treatment of "0.1 mg/L 2,4-D + 0.5 mg/L BAP" (with the highest values of the three traits studied) could be proposed to induce callus in *W. coagulans* and to achieve the biomass in higher levels.

The results of the current research, overall, revealed that for leaf tissue, the R1000 strain compared to the GM strain had a greater superiority for hairy root induction in *W. coagulans*. Instead, for stem tissue, inoculation with the GM strain apparently resulted in more hairy roots in this plant compared to the R1000 strain. Furthermore, it seems that “0.1 mg/L 2,4-D + 0.5 mg/L BAP” treatment is more suitable hormonal treatment for callus induction in *W. coagulans*, albeit, it may be different from one study to another one, possibly due to possible differences in the genetic backgrounds of the plant samples or other potential factors.

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