Journal of Ornamental Plants $www.iornamental. iaurusht.ac.ir$ **1SSN (Print): 2821-0093 ISSN (Online): 2783-5219**

Research Article Volume 13, Number 3: 169-179, September, 2023 **DOR**: https://dorl.net/dor/20.1001.1.28210093.2023.13.3.2.7

The Effects of Naphthaleneacetic Acid on the Rooting of Cycas *revoluta* Bulbs

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Received: 20 April 2023 **Accepted:** 14 August 2023

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The effects of naphthaleneacetic acid (NAA) $(0, 15, 20$ and 25 mg/l) were explored on the rooting of *Cycas revoluta* bulbs in a study based on a randomized complete block design with four replications. Based on the results, the NAA treatments significantly influenced root length, leaf fresh and dry weight, and chlorophyll. But, they did not affect root number, root fresh and dry weight, significantly. The longest roots were 9.43 and 9.63 cm produced by the bulbs treated with 25 and 20 mg/l NAA, respectively. The 25 and 20 mg/l NAA were related to the highest root number of 3.57 and 3.23, respectively, the 25 mg/l NAA was related to the highest mean root fresh weight of 4.9 g, the 25 mg/l NAA was related to the highest root dry weight of 2.5 g, the 20 and 25 mg/l NAA were related to the highest leaf fresh weight of 6.04 and 6.54 g, respectively, the 25 and 20 mg/l NAA were related to the highest leaf dry weight of 4.07 and 3.11 g, respectively, and the 20 and 5 mg/l NAA were related to the highest chlorophyll content of 6.28 and 6.22 mg g^{-1} F.W., respectively. A significant relationship was observed between leaf dry weight and chlorophyll content at the $P < 0.01$ level $(+0.78)$. But, there was no significant relationship between root dry weight and mean root length $(+0.29)$. The relationship between chlorophyll content and mean root length was not significant either $(+0.13)$. According to the results, increasing the hormone rate may even affect the plants adversely, injuring them. In general, it can be inferred from the results that NAA at the rate of 20 mg/l influenced the studied traits, thereby entailing the highest gene expression in C. *revoluta*.

Keywords: Chlorophyll, *Cycas revoluta*, Leaf fresh weight, Naphthaleneacetic acid, Root length.

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Abstract

Abstract

INTRODUCTION

Cycas is the only genus in the family of Cycadaceae with almost 100 species among which *Cycas revoluta* is the most famous (Agredano-Moreno *et al.*, 2022). *C. revoluta* is the second species in the genus cycas and is the only cycas that is distributed in Asia (Liu *et al.*, 1991). Cycas has a special botanical status since it is the dividing line of flowering and non-flowering plants. It has also been put in a separate group, named prophanrogam, in new taxonomies (Ghahraman, 2004). The plant has a very long history and is popular for its beautiful evergreen pinnate leaves. The species C. *revoluta* is used as an ornamental pot plant and an evergreen plant in green space design. The main habitat of C. *revoluta* is Southern Japan on 31°N including Ryukyu, Mitsuhama, and Satsuma in the South of Kyushu (Stevenson, 1990; Vozárová *et al.*, 2022). The species has been spread beyond Japan in cultivated form so that it is one of the mostly cultivated cycas species grown in open air in hot semi-tropical climates or in greenhouses in colder areas. In Iran, it can be cultivated in open air in the provinces of Guilan and Mazandaran. Cycas is propagated by seeds, offshoots, stem cuttings, and tissue culture. flowering plants. New taxonomies have put it in a separate group, prophanrogam. C. revoluta is *C. revoluta* has a special place in botany as it is the dividing line between flowering and nona popular green space species, which is used both as a leaf plant and in green space design due to its different engineering applications (Rouhani, 2010).

Auxins and their derivatives are involved in the formation and preservation of root apical meristem. The stabilization of the roots' quiescent center in natural roots is accompanied by maximum auxin concentration. Auxins induce the expression of genes WUS, WOX5, WOX, SHR, SCR, NPH4, MP, and PLT, contribute to the decomposition of IAA/AUX inhibitors, and disable the transcription regulation capability of ARFs by attaching them. Auxins create local symptoms that lead to specific transcription programs, which, in turn, mediate specific cellular behaviors that are involved in the formation and preservation of root apical meristems. There are factors that are involved in auxin mobilization and effective response on the formation of shoot apical meristems and cause auxin accumulation in apical regions at early embryogenesis stages and the polar distribution of PIN proteins, especially PIN1. There are also other factors involved, e.g., disrupting genes. These genes encode several distinctive groups of transcription factors, including the members of the families of DORNROSCHEN and HD ZIP 111 KANADI (Taiz and Zaiger, 2015). The expression of several root-specific WOX genes is sensitive to auxin. Some of these WOX genes, called WOX5, are expressed in a small group of root tip cells that contain the root quiescent center and its surrounding initials cells. The extremely centralized pattern of WOX5 expression is determined by the combined activities of the genes SHR, scheends to preserving the expression of WOX5 in the root quiescent center contributes to preserving these cells by preventing the premature differentiation of the surrounding initials cells. In the embryogenesis stages, the reversion of the distribution of PIN proteins results in the slow and directional redistribution of auxins. However, changes in the phosphorylation status of PINs, which is mediated by PINOID kinase and PP2 phosphatase, affect the local concentration of PINs significantly. A consequence of the complicated pattern of auxin mobilization in embryos is the formation of a central apical zone where auxin-dependent activities are less intense than those in marginal areas. The outflow of auxin from this zone is converged with upward superficial flows along the embryo sides so that maximum auxin occurs in the apical regions of the growing cotyledons. These auxin sinks enter the downward flows that converge at the axis under the cotyledon and then, the maximum auxin continues in the root quiescent center. ARFs,

e.g., NPH4 and closely related MP, are activated by auxin to improve vascular development and further reinforce this directional mobilization pattern. Mutants that concurrently lack NPH4 and MP not only lack basic structures such as roots but they also have no cotyledons. These phenotypes are similar to those that are related to mutations that influence PIN-mediated auxin mobilization and to the models in which NPH4 and MP allow auxin-dependent responses (Taize and Zaiger, 2015). Various genes are involved in the formation and preservation of shoot apical meristems. Screening for mutants that inhibit the formation of shoot apical meristems highlights three other groups of transcription factors. One of these groups is encoded by WUS and interestingly belongs to a similar family whose transcription factors have a protein range that includes WOX5. WUS is expressed in the subapical regions as early as the 16-cell embryonic stage and plays a key role in determining and maintaining the identity of apical primate cells of shoot apical meristems. The local expression of CUC genes and the subsequent emergence of STM show the relatively low level of auxin-dependent activities in flanking tissues. The inhibition of auxin signaling at the flanking colytedonary regions by the mutations of NPH4 and MP results in the expression of CUC genes outside their natural areas in these regions. The role of auxin signaling in natural embryos treated with auxin mobilization inhibitors is similar to its role in CUC-mutated embryos. The expression of quasi-CUC genes in the central apical area of the embryo creates an environment that allows further patterning processes, including the local expression of STM.

Auxins can influence leaf location. Leaf primordia can be stimulated for formation in unnatural areas on the apical shoot by applying auxin at slight quantities directly to the shoot apical meristem. The local concentration of auxin can be determined by DR5 reporters whose activity is closely linked with the leaf primate areas (Taiz and Zaiger, 2015). The location of leaf formation is linked with polar auxin transport patterns so that auxin transport patterns can be inferred from the asymmetrical location of PIN proteins. Leaf primordia initiate at the location of auxin accumulation. The movement of auxin to the tip is inhibited at the interface of the central and peripheral areas, resulting in increasing auxin concentration and initiating a leaf. The primordium that is formed acts as an auxin sink, so the immediate initiation of new leaves above it is inhibited. The presence of a more mature but farther leaf allows auxin transport to the tip, resulting in the initiation of another leaf. The growth of leaf blades is also auxin-dependent and is regulated by genes WOX and YABBY. The transcription factors of YABBY upregulate gene PRS (PRESSED), which is a family of WOX genes and is expressed at the periphery of the leaf, stimulating blade growth. The transcription factors of WOX1 and PRS cooperate. Blade growth is dependent on these two genes. The transcription factors WOX and PRS cooperate with each other and the growth of the stem depending on these two genes is partly processed by KL a cytochorom P450 mono oxygenase. KLU stimulates cell division activity in aerial parts, including leaves. A mutant that lacks KLU gene functioning grows smaller organs. Auxin is another signal involved in blade formation, but it is independent of KLU. Multiple mutants that lack the function of auxin biosynthesis genes YUCCA (YUC) lack blade growth and cooperate in directional leaf growth.

Auxin canalization initiates leaf development. Auxin also stimulates the formation of vascular tissue. The application of auxin to the cut petiole causes the regeneration of the vascular tissue as this regeneration needs auxin at the petiole. Regenerating xylem vessels originate from the auxin spot above the injury created in the vascular bundle and move downwards to attach the point below the lesion on the vascular tissue in order to restore the former path of auxin flow. The upper part of the lesion on the vascular bundle acts as a source of auxin and the lower part acts as a sink of auxin. A developing leaf is the auxin source, and the vascular tissue in the stem is the auxin sink. The downward auxin transport from the leaf primordium layer triggers leaf procambium development. Auxin canalization also regulates the formation of auxiliary veins. PIN1 guides auxin towards convergence points along the leaf margin in the epidermis of developing leaves. These convergence points are related to areas where the teeth and pores, called hydathodes, develop. As auxin concentration increases in these areas, auxin dispersion creates a PIN1-induced flow from the convergence points toward the main veins, which, in turn, causes the differentiation of pre-procambium along the direction that auxin flows until the secondary vein forms. Auxin transporters, e.g., ABCB 19, contribute to narrowing its path by inducing auxin outflow from surrounding cells, and AUX/LAX permeases create consumption sinks and increase auxin flow. The process of vein formation is as follows. Auxin is synthesized by YUCCA proteins and accumulates in hydathode regions. Then, the auxin flow from leaf margins induces PIN1 formation and its polar transport toward surrounding cells and the induction of auxin outflow from its synthesis spot. ABCB exporters increase canalization by inducing auxin outflow from all spots except for small areas, which results in the development of veins whereas LAX/AUX 1 absorption transporters create sinks that increase auxin flow. Then, auxin is absorbed by the developing cells of veins, which contributes to the preservation of auxin flow until the full differential of the veins (Taiz and Zaiger, 2015). The correct formation of adventitious roots at the base of stem cuttings is an important growth phenomenon for the growth and survival of cuttings, which includes the initiation of several new meristem areas in different tissues of stem cuttings (Kaur *et al.*, 2002). In addition, the adventitious root formation is generally extended by auxin, and auxin signaling and transport have been reported to control root length, the number of adventitious roots, capillary roots, and root growth direction (Li *et al.*, 2009). Auxin is a plant hormone that is involved in inducing rooting and the formation of root primordia. Nag *et al.* (2001) report that auxin is an essential factor for inducing roots instead of initiating roots in plants, which supports the hypothesis that adventitious roots first form in two steps $-$ an auxin-sensitive phase and an auxin-insensitive phase (Yan *et al.*, 2014). Naphthaleneacetic acid (NAA) is synthetic auxin that is usually applied at relatively low dosages to induce auxin-like responses in cell growth, cell division, fruit-bearing, rooting, and so on (Sun and Hong, 2010). NAA is considered a growth regulator that is involved in fertility development, cell division and elongation, and many physiological activities in plants (Sawan et al., 1998; Singh et al., 2017). The application of indole acetic acid $I(AA)$ increased plant growth significantly (Eifediyi and Remison, 2015). It was reported that the production of adventitious roots increased significantly at lower rates of NAA whereas the number of the roots decreased at higher rates (Saifullah and Bi, 2012). Despite the significance of plant propagation and cultivation, limited research has been conducted on the effect of NAA on ornamental plants. However, for competitiveness, countries need to adopt modern methods to shorten propagation time and enhance the quality of cuttings and plants. So, this research aimed to shed light on the effect of NAA on the growth and propagation of C. *revolute* and determine the best rate of NAA, thereby helping producers for competitive production of this plant species.

MATERIALS AND METHODS

The research was conducted in 2021 based on a randomized complete block design with four replications. The experimental treatments included naphthaleneacetic acid (NAA) at the

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rates of 0 (control), 15, 20, and 25 mg/l. The plant material was the bulbs of *Cycas revoluta*, which were evaluated in terms of root and shoot traits $(Fig. 1)$. All bulbs were planted in the same substrates with the same compositions $(40\%$ pumice $+ 60\%$ perlite). They all had similar porosity, water retention capacity, and cation exchange. The hormone treatments applied to the bulbs included NAA (Energil produced by Cifo, Italy) at the rates of 15, 20, and 25 mg/l and no-hormone application as the control. The hormone was first dissolved in distilled water, and it was then added to the water. It was then consumed as foliar application at the intervals of 15, 20, and 25 days in identical conditions at $>25^{\circ}$ C. The substrate's pH was in the range of 4-4.5 and its EC was estimated at about 1 mS. To measure the leaf fresh weight, some plants were randomly selected from each treatment. Then, their leaves were cut and cleaned to determine their weight. To measure the root dry weight, the selected bulbs were separated and cleaned. Next, they were oven-dried at 65°C for 48 hours. Finally, their weights were recorded. The leaf chlorophyll content was determined for each plot using a SPAD device.

Fig. 1. The rooted bulbs.

Data were analyzed using the SAS and SPSS statistical software packages. The means of the treatments were compared by the LSD test and Pearson's coefficients of correlation. Also, the graphs were all drawn using the MS-Excel software suite.

RESULT

percentage Rooting

In this research, 32 bulb were tested, and in 4 treatment, the control treatment had no rooting, and in the 15 ml treatment, rooting equal to 85.23 $\%$, and in the 20 ml treatment, we observed the highest percentage of rooting and in the last treatment which was 25 ml NAA, we had 83.34 % rooting. In total, we had 40.62 % rooting in 4 treatment and 79.17 % rooting in 3 hormonal treatment (Fig. 2). Hormonal treatments have increased the percentage of rooting and statistically, the difference between all 3 levels of naphthalene acetic acid hormone concentration of 20 ml treatment and the other two treatment compared to the control is statistically significant at the 5% level

Fig. 2. Bulbs of control treatment.

Root length

The effect of NAA was found to be significant $(P < 0.01)$ on root length (Table 1). It was revealed by the comparison of means that NAA at the rates of 25 and 20 mg/l was related to the highest root length of 9.43 and 9.63 cm, respectively. The lowest root length was 3.49 cm related to the control (Table 2). With increasing the auxin concentration, the expression of two PLT genes is activated, subsequently resulting in the activation of NPH4, MP, SCR, and SHR. The integrated expression of these genes leads the cells in the center toward becoming the quiescent center and also stimulates the expression of WOX5, which is involved in the preservation of surrounding primate cells. This gene plays an important role in the root apical *neristem* (Taiz and Zaiger, 2015). This finding is consistent with Habibi (2012). Pourdanesh *et al.* (2015) reported that the effect of NAA was significant on the rooting of *Cupressus macrocarpa* cuttings. According to their results, NAA also influenced root length, rooting percentage, and root fresh and dry weight. In a study on the effect of indole butyric acid (IBA) and NAA on the rooting of *Camellia japonica* L. cuttings, Hashemabadi and Sedaghathoor (2007) reported that NAA had significant effects on rooting percentage, root dry matter, root length, longest root length, and root number. Sun *et al.* (2023) also used hormone concentrations of 800, 500, 200 $& 1000$ mg/l of NAA hormone in an experiment. This test was for the propagation of murus cutting, and the longest root was obtained with a concentration of 800 mg/l. The longest root length (10.20 cm) and average root length (4.44 cm) of cuttings achieved the best results when soaked with 800 mg/l NAA for 60 min and 500 mg/L NAA for 30 minute (Sun *et al.*, 2023).

* and ^{ns}: Significant at $P \le 0.05$, $P \le 0.01$ and insignificant based on the LSD test, respectively.

number Root

The analysis of variance showed that the effect of NAA was not significant on root number (Table 1). It was revealed by the comparison of means that NAA at the rates of 25 and 20 mg/l was related to the highest number of roots $(3.57 \text{ and } 3.23$, respectively), but not differing from the other treatments significantly (Table 2). These results are not consistent with some other research (Hashemabadi and Sedaghathoor, 2007). Habibi (2012) reported that NAA influenced traits like root length, rooting percentage, the longest root length, and root dry weight significantly. According to the results, the best NAA concentration was 300 mg/l .

Treatments	Root length (cm)	Root no.	Root F.W. (g)	Root D.W. (g)	Leaf F.W. (g)	Leaf D.W. (g)	Chlorophyll (mg/ml)	Mean root length (cm)
Control	3.49 _b	2.99a	1.98 a	1.04a	2.43 _b	1.24 _b	4.67 ab	1.19 _b
25 mg/l NAA	9.43a	3.57 a	4.51a	2.27a	6.04a	4.07a	6.28a	2.65a
20 mg/l NAA	9.63a	3.23a	4.9a	2.5a	6.54 a	3.11 a	6.22a	3.03a
15 mg/l NAA	4.65 b	2.28a	3.16a	1.62a	1.29 _b	0.65 _b	1.65 _b	2.13 _b

Table 2. The comparison of means for the studied traits

 $*$ In each column, means with similar letter(s) are not significantly different ($P < 0.05$) using the LSD test.

Root fresh and dry weight

The treatment of NAA could not influence root fresh and dry weight significantly (Table 1). The comparison of means showed that the plants treated with 25 mg/l NAA had the highest root fresh weight of 4.9 g, but it did not exhibit any significant differences with the other levels of NAA (Table 2). The highest mean root dry weight was 2.5 g related to the treatment of 25 mg/l NAA (Table 2). These results are inconsistent with Rahdari *et al.* (2010) who studied the effect of zinc sulfate on NAA and IBA hormones in *Aralia elegantissima* and revealed that the effect of the growth hormones was significant on the recorded traits including dry weight, the bercentage of rooted cuttings, fresh weight, root number, and root length.

Leaf fresh weight

The treatment of NAA influenced leaf fresh weight significantly at the $P < 0.05$ level (Table 1). The plants treated with 25 and 20 mg/l NAA produced the highest leaf fresh weight of 6.04 and 6.54 g, respectively. They were categorized into group a in the table of means hardwood cuttings of rosemary, Shahhoseini et al. (2015) reported that different levels of the comparison (Table 2). In similar results in a study on the effect of NAA on the rooting of semigrowth regulators were significant for the studied traits including plant dry and fresh weight. The increase in auxin concentration resulted in the expression of WOX. This gene is essentially involved in the formation of shoot apical meristem. Genes belonging to this family contain a distinctive from of the homebox DNA binding motif. WUS is another gene that is required for both the formation and the preservation of shoot apical meristem and seems to be similar to PLT genes. This gene acts at the shoot apical meristem to maintain the population of undifferentiated primate cells. A certain sequence of gene expression is involved in the formation of the apical area, including the expression of WUS, which induces CLV3 expression in the external adjacent cell layers, and the expression of ANT, which is an indicator of leaf or cotyledon identity. As such, auxin helps growth, which subsequently increases the weight of all plant organs (Taiz and Zaiger, 2015).

Leaf dry weight

The results of variance analysis revealed the significant effect of NAA on leaf dry weight at the $P \le 0.05$ level (Table 1). It is observed that the NAA rates of 25 and 20 mg/l were related to the highest leaf dry weight of 4.07 and 3.11 g, respectively, so they were put in group a (Table 2). Hassanzadeh *et al.* (2017), who studied the yield of quality of okras, reported similar results. They found that NAA had a significant effect on leaf dry weight and dry matter percentage in okra plants. In a similar study, Salamatmanesh and Vahdatifar (2010) , who investigated the effect of auxin type and concentrations on the rooting of *Thymus daenensis* Celak, found that the hormones of the auxin group influenced total dry weight, total fresh weight, stem fresh weight, stem dry weight, and root number significantly. They reported the hormone rate of 2000 mg/l to be the best for the studied traits.

Chlorophyll content

Chlorophyll content was significantly ($P < 0.01$) affected by the NAA treatments (Table 1). The plants treated with 25 and 20 mg/l NAA exhibited the highest chlorophyll contents of 6.28 and 6.22 mg/ml, respectively, and were put in group a (Table 2). Hassanzadeh *et al.* (2017) reported the significant effect of NAA on the total chlorophyll content, yield, and quality of okras. They obtained the highest total chlorophyll content from the NAA rate of 25 mg/l. Fateh and Barzegar (2019) studied the effect of the foliar application of NAA on the growth, yield, and quality of bell pepper cy. 'California' fruits and found that the treatment influenced vegetative traits, chlorophyll content, yield, yield components, and fruit quality. The highest yield was obtained from the NAA rates of 50 and 100 mg/l. They also reported that the treatment of 150 mg/l NAA was associated with the highest level of chlorophyll. In a study on the effect of different levels of NAA on the growth, yield, and quality of okra cv. 'Kano', Hassanzadeh *et al.* (2017) found that the highest levels of traits like total chlorophyll content, leaf number, plant height, leaf area, total yield, dry matter percentage, 1000-seed weight, and fruit number were obtained from the NAA level of 25 mg/l and the lowest from the NAA level of 100 mg/l. According to these authors, increasing NAA resulted in the loss of growth traits and yield, which agrees with our findings.

Mean root length

The effect of NAA was found to be significant ($P < 0.05$) on the mean root length (Table 1). The comparison of means revealed that the NAA rates of 25 and 20 mg/l were related to the highest mean root lengths of 2.65 and 3.03 cm, respectively. They were put in group a (Table 2). This is consistent with Shazly et al. (2001) who reported that the use of NAA stimulated adventitious roots and dormant root primates and was effective in increasing root length.

Correlation coefficients of the studied traits

According to the results, root length had significant relationships with root fresh weight (0.81) , root dry weight (0.8) , leaf fresh weight (0.8) , leaf dry weight (0.62) , and mean root length $(+0.78)$ at the $P < 0.01$ level. There was also a significant relationship between root length and root number (+0.53) at the $P < 0.05$ level. However, the relationship between root length and chlorophyll content was not significant $(+0.46)$ (Table 3). Root number was significantly related to root fresh weight $(+0.62)$, root dry weight $(+0.68)$, and leaf fresh weight $(+0.63)$ at the $P < 0.01$ level and leaf dry weight (+0.6) and chlorophyll content (+0.58) at the $P < 0.05$

level. But, no significant relationship was observed between root number and mean root length (-0.07) (Table 3). The relationship of root fresh weight was significant with root dry weight $(+0.99)$ and leaf fresh weight $(+0.66)$ at the P < 0.01 level and with leaf dry weight $(+0.54)$ at the $P < 0.05$ level. It, however, had no significant relationship with chlorophyll content $(+0.32)$ and mean root length $(+0.47)$ (Table 3). The relationship between root dry weight and leaf fresh weight (+0.65) was significant at the $P < 0.01$ level, and the relationship between root dry weight and leaf fresh weight $(+0.53)$ was significant at the $P < 0.05$ level. But, the relationship of root dry weight was not significant with chlorophyll content $(+0.32)$ and mean root length (-0.45) . A significant relationship was detected between leaf dry weight and chlorophyll content $(+0.78)$ at the P < 0.01 level. No significant relationship was, however, detected between root dry weight and mean root length $(+0.29)$ and between chlorophyll content and mean root length $(+0.13)$ (Table 3). These results corroborate Hashemabadi and Sedaghathoor's (2007) report about camellia, Shahhoseini *et al.*'s (2015) report about rosemary, and Soost *et al.*'s (2006) report about 14 peach cultivars.

	Root length	Root no.	Root F.W.	Root D.W.	Leaf F.W.	Leaf D.W.	chlorophyll content	Mean root length		
Root length	1	$+0.53^*$	$+0.81**$	$+0.8***$	$+0.8***$	$+0.62$ **	$+0.46$ ^{ns}	$+0.78**$		
Root no.			$+0.62$ **	$+0.68$ **	$+0.63**$	$+0.6^*$	$+0.58*$	-0.07 ^{ns}		
Root F.W.				$+0.99**$	$0.99**$	$0.54*$	$+0.32$ ^{ns}	$+0.47$ ^{ns}		
Root D.W.					$0.65***$	$+0.53*$	$+0.32$ ^{ns}	$+0.45$ ^{ns}		
Leaf F.W.						$0.87**$	$0.79**$	$+0.46$ ^{ns}		
Leaf D.W.							$0.78**$	$+0.29$ ^{ns}		
Chlorophyll								$+0.13^{ns}$		
Mean root length										

Table 3. The coefficients of correlation between the studied traits.

*, ** and ^{ns}: Significant at P < 0.05, P < 0.01 and insignificant based on the Pearson's coefficients of correlation, respectively.

CONCLUSION

Rooting in cutting of woody species is hard and time consuming. We tried to induce root formation on cycad (*Cycas revolute*) bulb. The results showed that the effect of NAA was significant on measured traits including rooting percentage. Maximum rooting percentage and root number was obtained using 20 and 25 ml NAA, respectively. Treatment of 20 ml NAA for efficient and economical rooting of *C. revolute* bulb is suggested. Future research should to be directed towards the use of lesser-Known plant growth regulators particularly auxins to enhance rooting of hard-rooted cutting.

ACKNOWLEDGMENT

Thank you to all the people who helped in this research.

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Asgari Gouraj, M. (2023). The Effects of Naphthaleneacetic Acid on the Rooting of Cycas revoluta Bulbs. Journal of Ornamental Plants, 13(3), 169-179.

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How to cite this article: