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Spirulina plantensis Extract: Natural Growth Regulator of *in vitro* Paulownia Cultures

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ABSTRACT

Paulownia is one of the most industrial trees nowadays concerning wood production. *In-vitro* culture of *Paulownia* will improve the quality of the produced wood otherwise the possibility of using it for medicinal purposes with various contained bioactivities compounds. This study focused on developing a new *in vitro* line of *paulownia* hybrid (*Paulownia Shan Tong*) with the highest content of bioactive compounds and recorded the highest growth rate by using Spirulina extract as a natural growth regulator rather than synthetic hormones. Efficient Germination and shoot induction protocols were conducted. Different treatments were tested, and P6 treatment (6.0 mg/L of AE extract) was recorded. The Highest shoot length was recorded at 4.4±0.41cm, leaves number 6.8±0.37 (L/Explant), the highest root length at 8.0±1.0 cm, and the maximum root number at 5.0± 0.31 (R/Explant). The total phenolics and total flavonoids analyses recorded 365.42±5.69 mg GAE/g and 242.5±5.75 mg QE/g, respectively. Root in medium contained 6.0 mg/L of AE extract induced strong branched roots facilitated the acclimatization stage. This efficient protocol for *paulownia in vitro* production can be applied for commercial tissue culture laboratories and presents a new trend for *in vitro* economic woody plant production with low-cost.

Keywords: *Paulownia, Spirulina plantensis*, phytohormones, GRs.

1. Introduction

Spirulina platensis serves as a valuable source of both macro- and micronutrients for plants, providing components such as vitamins, amino acids, polypeptides, and phytohormones like gibberellins, auxins, and cytokinins (Bhowmik *et al.,* 2010; Osman *et al.,* 2016 and Nawrocka *et al.,* 2017). *Paulownia* species are recognized as medicinal plants (Zhu *et al.,* 1986; Yadav *et al.,* 2013 and Wo´zniak *et al.,* 2019). Their extracts can be utilized for treating gonorrhea and bruises, promoting hair growth, and preventing hair graying (Yamauchi *et al.,* 1987). Traditional Chinese medicine employs various preparations derived from the bark, fruit, xylem, or leaves to address numerous ailments, including hemorrhoids, inflammatory bronchitis, gonorrhea, parotitis, asthma, erysipelas, bacterial diarrhea, bronchopneumonia, enteritis, hypertension, and tonsillitis (Si *et al.,* 2012). Multiple phytochemical investigations have demonstrated that *paulownia* species generate a variety of secondary metabolites, with polyphenolic compounds being particularly significant due to their strong antioxidant abilities (Durán Zuazo *et al.,* 2013 and Smejkal *et al*., 2007). Pharmacological research has also shown antioxidant, anti-inflammatory, and antimicrobial effects against Staphylococcus aureus and Pseudomonas aeruginosa (Kim *et al.,* 2000 and Jo *et al.,* 2019). Nevertheless, the chemical makeup and biological attributes of *paulownia* species remain largely unstudied. Only a limited number of compounds have been recognized, primarily in the flowers, which include phenolic glycosides, furofuran lignanes, furanoquinones, iridoids, and flavonoids (Smejkal *et al*., 2007; Jiang *et al.,* 2004 and Oprea *et al.,* 2004). Within the secondary metabolites, phenylethanoids and phenylpropanoids hold significant importance, with verbascoside (acteoside) and its isomers and derivatives being the most prevalent representatives (Smejkal *et al*., 2007 and Schneiderová *et al.,*

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2015). This study aimed to improve the *in vitro* production of paulownia hybrid **(***Paulownia Shan Tong***)** using spirulina algae extracts as a natural source of bioactive compounds that can be used instead of the usual expensive hormones for commercial production.

2. Material and Methods

2.1. Plant material and Sterilization

The mature seeds of *paulownia* hybrid **(***Paulownia Shan Tong***)** were sterilized by immersion in 70% ethyl alcohol for 1 min followed by solution of 0.1% (w/v) mercuric chloride ($HgCl_2$) for 2 minutes, then seeds washed three times. The washed seeds were immersed in 30% solution of sodium hypochlorite for 10 minutes containing 5 drops of Twin-20. Finally, seeds were finally washed for 3 times using sterile distilled water.

2.2. Germination and Seedling Condition

Paulownia seeds were germinated on germination media MS (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 7 g/L agar for solidification and for promoting germination of seeds 2.0 mg/L of Gibbrelic Acid (GA3) were added, pH was adjusted to 5.7. Three replicates for each treatment were conducted. All seed cultures were incubated in growth chamber at 25 °C ±1 °C in the dark for 4 weeks.

2.3. Shooting Induction and multiplication of *Paulownia* Hybrid

The seedlings were separated and cultured on a shooting induction medium containing 1.0 mg/L Indole Acetic Acid (IAA) and incubated for one month under a photoperiod of 16 h light and 8 h of dark at 22±2°C, then the obtained shoots were cut into segments of 0.5 cm length, each with at least one nodal section. The nodal segments were transferred on MS media provided with different combinations of growth regulators (Benzyle adenine (BA), Thidizuron (TDZ), and Indole Acetic Acid (IAA)) as reported in Table (1).

Table 1: Composition of media for multiplication of *Paulownia* hybrid (*Paulownia Shan Tong*)

Treatments	Growth Regulators (mg/L)		
	BA	**TDZ**	**IAA**
T0	0.0	0.0	0.0
T1	0.25	0.0	1.0
T2	0.5	0.0	1.0
T3	1.0	0.0	1.0
T4	0.0	0.25	1.0
T5	0.0	0.5	1.0
T6	0.0	1.0	1.0

2.4. Treatments of Algae Extract (AE) on *Paulownia* hybrid (*Paulownia Shan Tong*)

Shoot tips were separated from the regenerated shoots and cultured on AE treatments (MS basal medium supplemented with different concentrations of Spirulina Algae extract (Table 2). Each treatment was replicated for statistical analysis, under incubation condition of photoperiod of 16 h light / 8 h dark at 22±2°C. After 6 weeks, the data on the growth rate were recorded and statically analyzed.

Table 2: Different treatments of algae extract (AE)

Treatments	Concentrations of spirulina algae extracts(AE) (mg/L)
P0	0.0
P1	1.0
P2	2.0
P3	3.0
P4	4.0
P5	5.0
P6	6.0
P7	7.0
P8	8.0
P9	9.0

2.5. Rooting Culture

For rooting, the obtained shoots were cultured on a modified medium containing MS basal medium and 10 g/L sucrose, supplemented with 0.25mg/L Indole-3-butyric acid (IBA) in combination with 6.0 mg/L of spirulina algae extract and incubated for one month under photoperiod condition of 16 h light/ 8 h dark at 22±2^{o}C.

2.6. Acclimatization of *Paulownia* hybrid (*Paulownia Shan Tong*)

Induced-rooted shoots were transferred from the modified rooting medium for the acclimatization stage in the green greenhouse. A mix of sterilized peat and sand in a volume ratio of 2:1 was put in small plastic pots for culturing the plantlets. Roots of the induced rooted shoots were gently washed with water to eliminate any residues of the modified rooting medium. After plantation in the prepared pots, all pots were covered with transparent polyethylene bags for four weeks. For plantlet adaptation, the polyethylene bags were gradually removed. The pots were incubated in the greenhouse at 22 °C under diffuse light conditions 16/8 h photoperiod/60 µMm-2s-1 and relative humidity around 95%. The humidity was reduced gradually and the plantlets were transferred to the field after 4 weeks to grow under sunlight.

2.7. Determination of total phenolic contents

For determination of total phenolic contents, shoots of the best Algae extract experimented treatment were separated for extraction as well as shoots from control treatment MS medium (free AE). The extraction method was conducted according to Dżugan *et al.* (2021), Total phenolic contents was determined using Folin–Ciocalteu reagent (Agbor *et al.,* 2014). The phenolic content was assessed as mg of Gallic acid (GAE) equivalents/gram of plant dry mass (mg GAE/g) was calculated based on a calibration curve for Gallic acid [0–250 _g/mL (y = 0.0555x, R2 = 0.9976)].

2.8 Determination of total flavonoid contents

To determine and assess total flavonoids, the method by Biju *et al.* (2015) was conducted. The total content of flavonoids in the extracts was expressed in mg of quercetin equivalent (QE) per gram of plant dry mass (mg QE/g). The results were calculated based on a calibration curve for quercetin [0–125 _g/mL (y = 0.0655x, R2 = 0.9999)].

2.9. Statistical Analyses

The statistical analyses were conducted using IBM®SPSS® [SPSS Inc; IBM Corporation, NY, USA] Statistics program, Version 25 (2017), Windows. Data were tested for normal distribution by Shapiro-Wilk's test (Shapiro and Wilk 1965; Razali and Wah, 2011). Data were inserted into ANOVA with a P-value of <0.05. The averages of all treatments were compared by the least significant difference as a post-hoc test (Snedecor *et al.,* 1956), P-value of <0.05 as being considered statistically significant (Alaa *et al.,* 2021).

3. Results and Discussion

3.1 Germination of *Paulownia* hybrid (*Paulownia Shan Tong*).

The germinated seeds were initiated on MS medium containing 2.0 mg/L of Gibbrelic Acid (GA3) as observed in Fig (1.a). The germination medium was selected based on different germination treatments and were previously experimented on different varieties of *Paulownia* (Data not shown), the selected medium resulted in the highest percentage of germination 87%.

3.2 Effects of GRs on Propagation of *Paulownia* hybrid

The regenerated shoots emerged from the nodal segments after 2 weeks of culture and the shoots were elongated to up to 2 cm in length which was suitable for the separation of shoot tips. The highest length and number of shoots per explant were recorded on T2 treatment at 0.5 mg/L BA and 1.0 mg/L IAA (Fig 1.B), this result agrees with the other study by Lydia *et al* 2014. The combination between BA and IAA (T2), increased shoot length and shoot number compared to the other experimented treatments with TDZ which was mentioned in several studies as an efficient regulator for most of the *in vitro* cultures (Murthy *et al.,* 1998; Saxena *et al.,* 1992; Guo *et al.,* 2011 and Corredoira *et al.*, 2008). The effects of the shooting induction treatments on shoot length are shown in Table (3).

Table 3: Effects of GRs on the shoot Induction of *Paulownia* hybrid (*Paulownia Shan Tong*).

Treatments	Shoot length(cm)	Shoot Number
T0	0.75±0.38	1.0±0.0
T1	1.75±0.14	2.0±0.57
T2	3.7±0.46	4.33±0.33
T3	3.08±0.60	3.33±0.33
T4	2.73±0.30	1.66±0.33
T5	2.013±0.18	2.6±0.33
T6	2.3±0.33	2.3±0.33

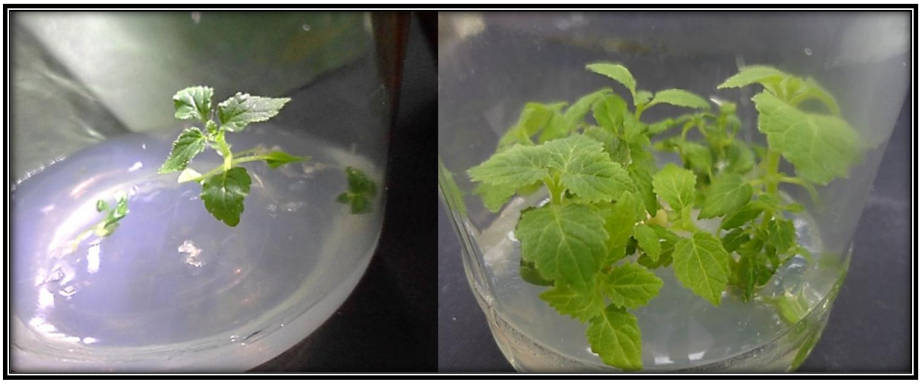

Fig 1: Germination and shoot propagation of *paulownia* hybrid (*Paulownia Shan Tong*). A. germinated seeds cultured on the GM medium, B. propagated shoots cultured on T2 medium.

3.3 Effects of Algae Extract on Growth rate of *Paulownia* hybrid:

Spirulina Algae Extract known as green blue algae is very nutritious and rich in antioxidants to help support a human's health, recently scientific research approved that its contents of plant hormones positively affected *in vitro* plant growth, the previous study by Marwa and Entesar. (2022) recorded the presence of gibberellins at 241.6, kinetin at 150.2 and Adenine at 140.6 ppm in *Spirulina platensis* extract. The previous study by Amin *et al.* (2009) verified the existence of phytohormones involving indole acetic acid, cytokinin, and gibberellin in the aqueous extract of Spirulina, This study aimed to apply this bioactive natural extract instead of usual growth regulators to develop the growth rate of *Paulownia* hybrid and to present a new natural growth regulator that can be used in different concentrations for targeting shoot induction, propagation, and rooting of the hardest *in vitro* cultures

of woody plants. Different concentrations of AE extract were tested as mentioned in Table (2). P6 treatment with 6.0 mg/L of AE extract recorded the highest shoot length at 4.4±0.41 (Fig 2), the highest root length at 8.0±1.0 (Fig 4) and maximum root number at 5.0±0.31 (Fig 5). The number of leaves increased at 7.0±0.31 with 0.3 mg/L (Fig 3) while recorded 6.8±0.37 with 6.0 mg/L of AE extract, little difference between two concentrations P3 (3.0 mg/L) and P6 (6.0 mg/L) concerning number of leaves (Fig 3). Some morphological changes appeared concerning shoot length, number of leaves and leave diameter due to increasing AE concentration up to 7.0 mg/L (Figs. 6.J and 7.K) Otherwise, the adequate concentration for shoot and root induction of *Paulownia* hybrid (*Paulownia* Shan Tong) was at 6.0 mg/L AE extract as shown in Fig (6.G).

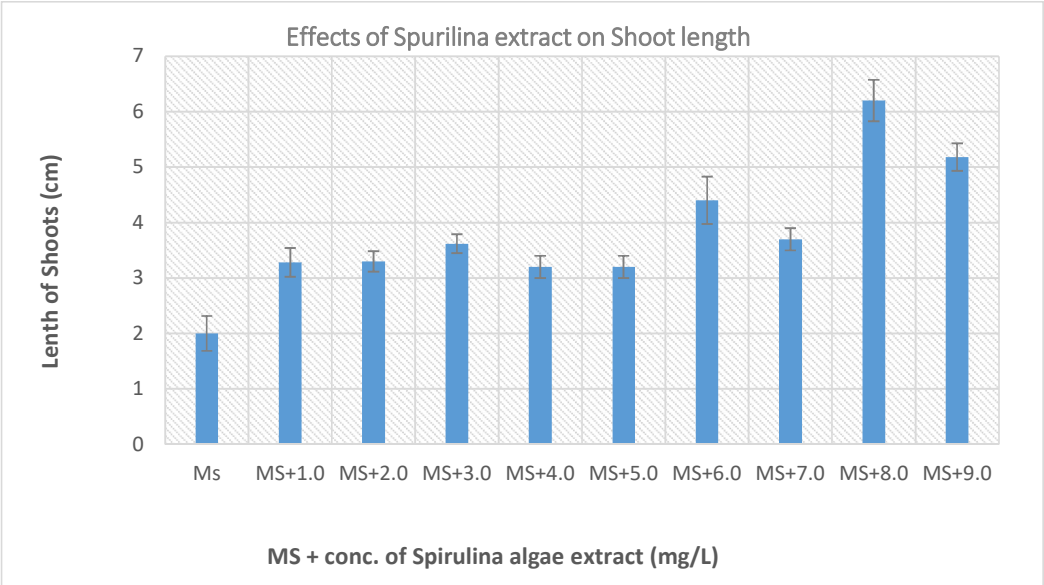

Fig. 2: Effects of different concentrations of algae extract (AE) on shoot length of *Paulownia* hybrid (*Paulownia Shan Tong*).

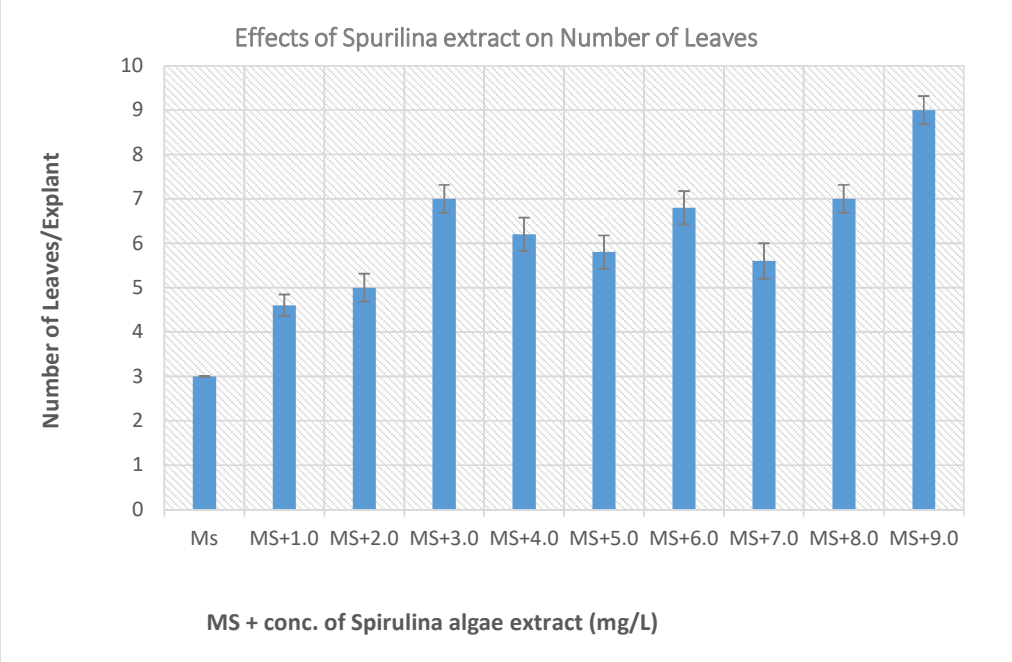

Fig. 3: Effects of different concentrations of algae extract (AE) on Leaves number of *Paulownia* hybrid (*Paulownia Shan Tong*).

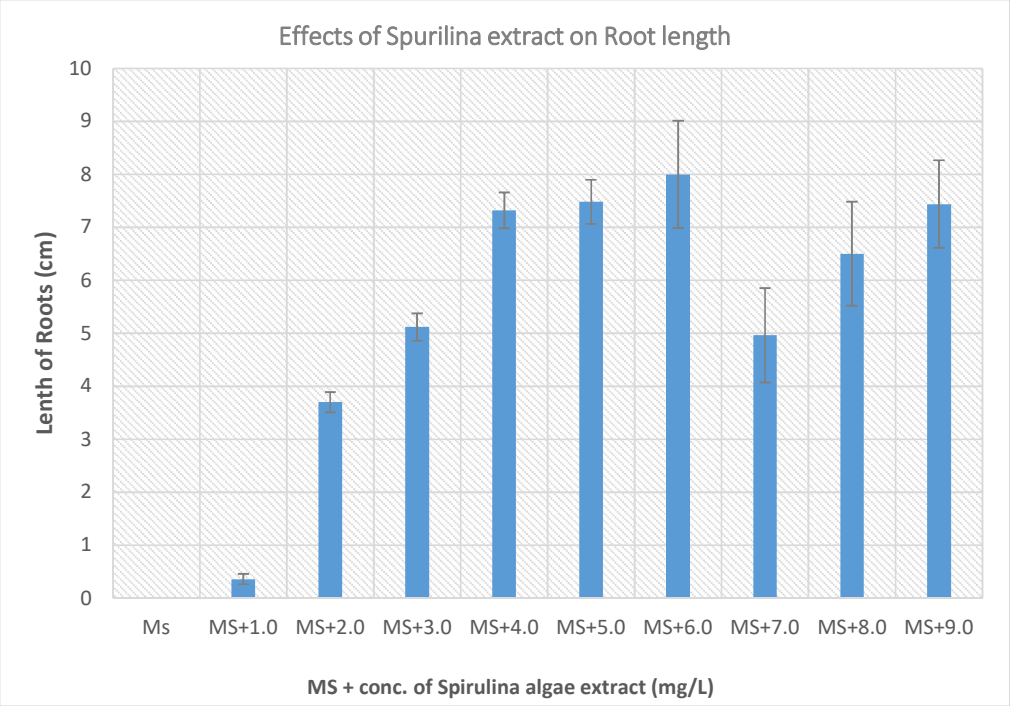

Fig. 4: Effects of different concentrations of algae extract (AE) on root length of *Paulownia* hybrid (*Paulownia Shan Tong*).

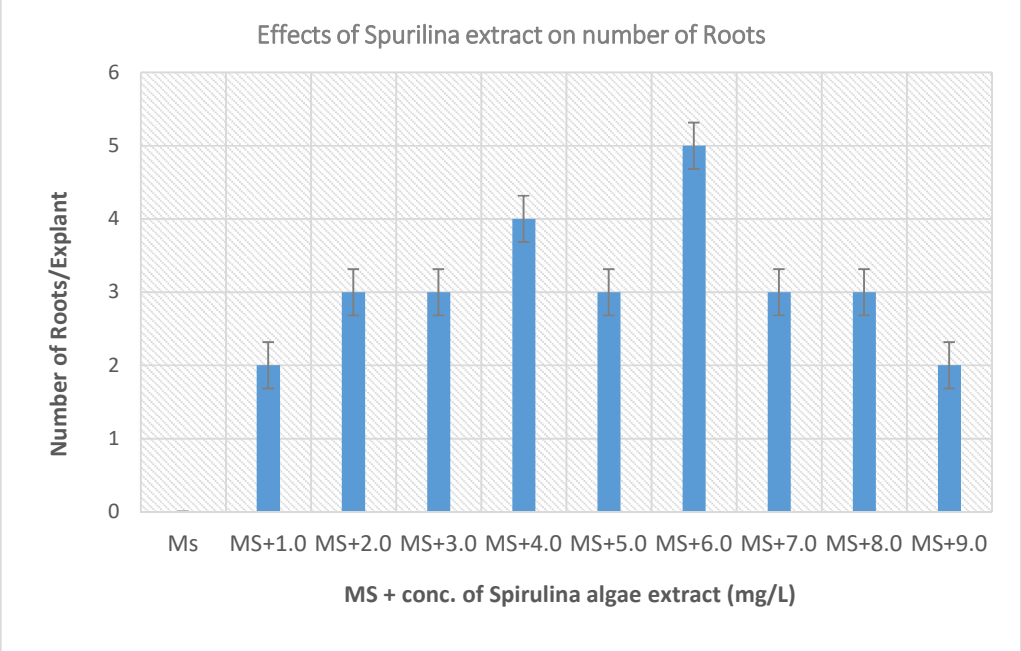

Fig. 5: Effects of different concentrations of algae extract (AE) extract on Root Number of *Paulownia* hybrid (*Paulownia Shan Tong*).

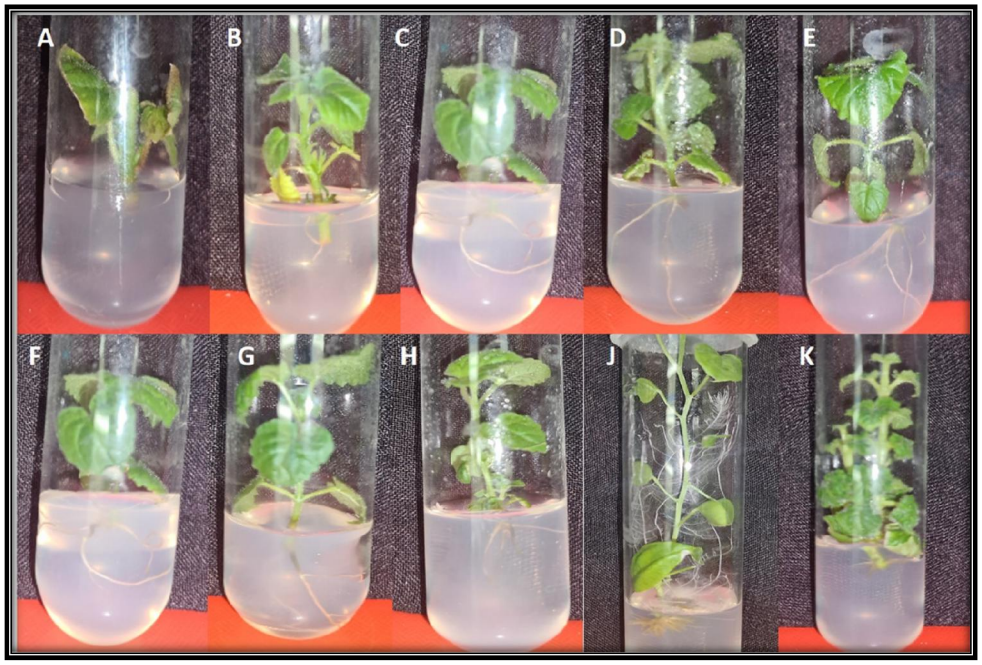

Fig. 6: Morphological effects of different concentrations of spirulina Algae extracts on *paulownia* hybrid *Shan Tong* A: control P0 (MS)*;* B: P1 (MS+ 1 mg/l AE)*;* C: P2 (MS+ 2 mg/l AE); D: P3 (MS+ 3 mg/l AE); E: P4 (MS+ 4 mg/l AE); F: P5 (MS+ 5 mg/l AE); G: P6 (MS+ 6 mg/l AE); H: P7 (MS+ 7 mg/l AE); J: P8 (MS+ 8 mg/l AE) and K: P9(MS+ 9 mg/l AE).

3.4. Total phenolic and Total Flavonoids

The determination of total phenolics and total flavonoids was conducted to study the effects of the best AE treatment on the bioactive compounds in the *in vitro*-produced *paulownia* hybrid. The development of plant growth was reflected positively as well in the phenolic and flavonoid content of *paulownia* (Fig 7.A) showing the difference between the total phenolic content at 365.42±5.69 mg GAE/g in the treated plant extract (produced from shoots cultured on P6) and at 226.76±4.87 GAE/g in control plant extract (produced from shoots cultured on P0 (MS free)). The total flavonoids recorded 242.5±5.75 mg QE/g in the treated plant extract (produced from shoots cultured on P6) and recorded 131.9±5.75 mg QE/g in the control plant extract (produced from shoots cultured on P0 (MS free) (Fig 7.B). These promising results present a new growth regulator isolated from nature with low cost to be applied in tissue culture laboratories for scientific and commercial purposes.

3.5 Rooting and Acclimatization

The plantlets were rooted perfectly after culturing on the modified rooting medium with 0.25mg/L IBA in combination with 6.0 mg/L AE extract (Fig 8.A). The strong induced roots (Fig 8.B) facilitated the acclimatization stage after culturing under greenhouse conditions. Plants were acclimatized faster than control plants which were not treated with any growth regulator or AE extract, Nutrients and growth hormones contained in AE extract provided plants with more beneficial elements to resist the environmental shock after a long period of incubation under aseptic conditions with a higher percentage of humidity (Fig. 9).

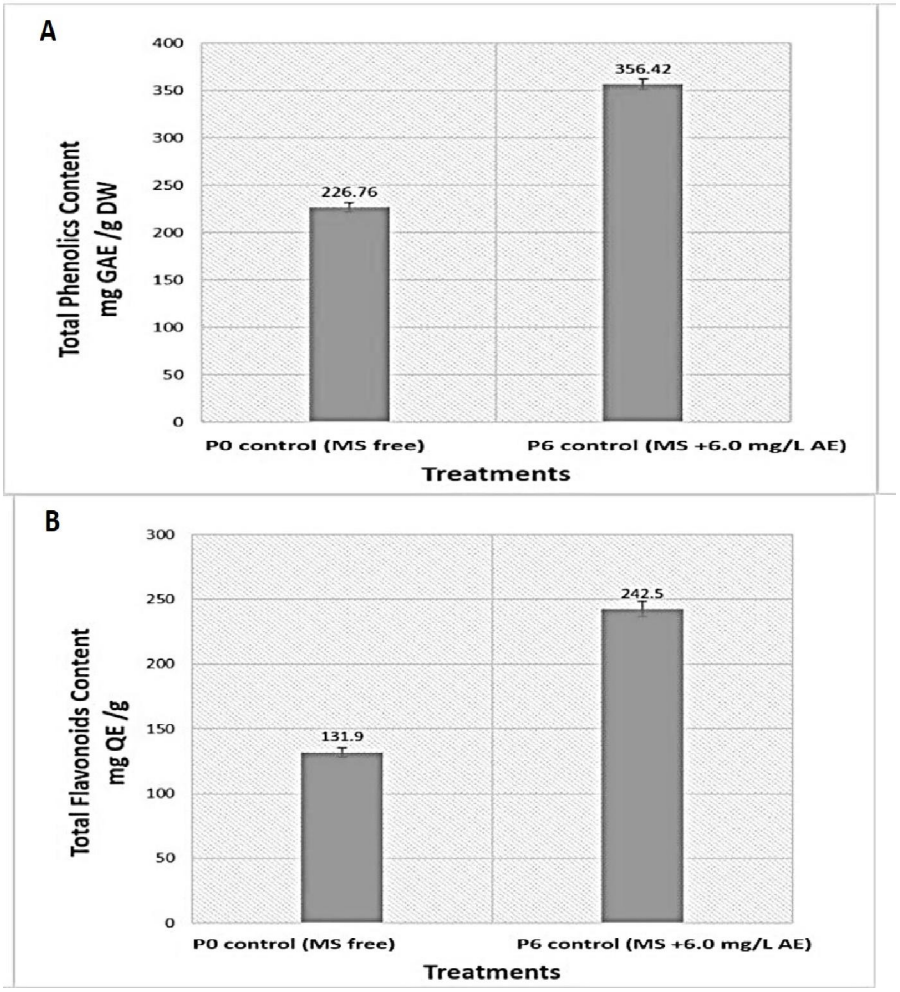

Fig. 7: Effects of P6 treatment on bioactive compounds in *paulownia* hybrid **(***Paulownia Shan Tong***)** extracts. P0: control, P6 treated with AE Extract at 6.0 mg/L. A. Total phenolic content. B. Total flavonoids content

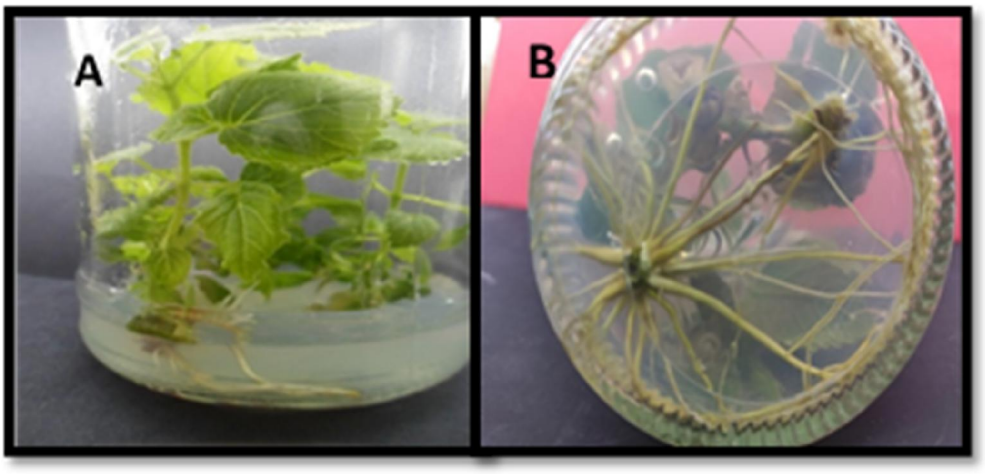

Fig. 8 A. B: Rooting culture of *paulownia* hybrid on the modified Rooting medium.

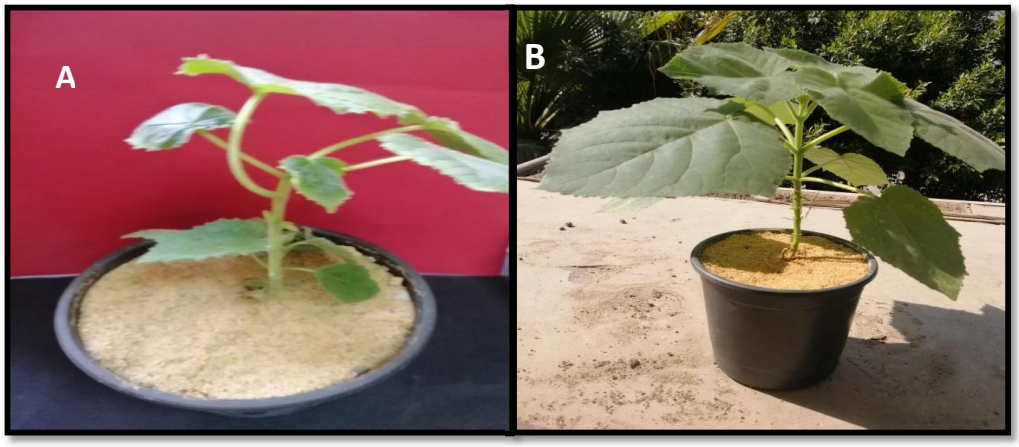

Fig. 9. A: Adapted *Paulownia* hybrid in greenhouse after the first month of acclimatization .B. Adapted *paulownia* hybrid under sunlight after two month of acclimatization

Conclusion

This study reveals the potential of using the Alga extract (*Spriulina Plantensis)* as a growth regulator for the induction of shoots and roots moreover the enhancement of bioactive compounds in *paulownia* hybrid **(***Paulownia Shan Tong***)**. The data recorded the adequate concentration at 6.0 mg/L of Algae extract with total phenolics at 365.42±5.69 mg GAE/g and Total Flavonoids at 242.5±5.75 mg QE/g. These results represented a suitable *paulownia* propagation protocol.

Conflict of interest

The authors have declared no conflict of interest.

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