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Plant Regeneration from Leaf Explants of the Medicinal Herb *Wedelia chinensis*

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Abstract: *Wedelia chinensis*, belonging to the Asteraceae family, has been used in folk medicine in East and South Asia for the treatment of common inflammatory diseases and protection against liver toxicity. Previously, in vitro propagation through different tissue explants has been reported, including through nodal segments, axillary buds, and shoot tips, whereas leaf segments failed to proliferate. Here, we report on the in vitro propagation of *W. chinensis* by culturing young leaf explants in MS medium supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA), 0.75 mg/L thidiazuron (TDZ), 1 mg/L gibberellic acid (GA_3), 3.75 mg/L adenine, 3% sucrose, and 0.8% agar at pH 5.8. Calli were observed in all explants derived from the youngest top two leaves, and the average percentage of shoot regeneration was 23% from three independent experiments. Then, several shoots were excised, transferred onto MS basal medium supplemented with 3% sucrose and 0.8% agar at pH 5.8, and cultured in a growth chamber for 1 to 2 months. Roots were easily induced. Finally, plantlets carrying shoots and roots were transferred into soil, and all of them grew healthily in a greenhouse. No morphological variation was observed between the regenerated plantlets and the donor wild-type plants. In addition, we also established root cultures of *W. chinensis* in culture medium (MS medium, 3 mg/L NAA, 3% sucrose, pH 5.8) with or without 0.8% agar. To the best of our knowledge, this is the first paper reporting plant regeneration from leaf explants in the herbal plant *W. chinensis*.

Keywords: in vitro propagation; medicinal plant; plant tissue culture; root culture

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1. Introduction

Medicinal plants are of great importance due to their potential uses in treating diseases through traditional medicine and in the pharmaceutical industry. *Wedelia chinensis*, belonging to the family Asteraceae, is a medicinal plant that has been used in traditional medicine for a long time. It is a procumbent and perennial herb found in wet and marshy places in East and South Asia. This plant has anti-inflammatory activity and has been used as alternative remedy to treat many infectious diseases, hepatic disorders, fever, cough, phlegm, cephalalgia, and skin diseases as well as being used in promoting hair growth, dyeing, etc. [1]. Protection against liver toxicity has also been reported [2,3]. Leaves of *W. chinensis* and *Eclipta alba* contain the coumestan compounds wedelolactone and demethylwedelolactone and have an anti-hepatoxic effect in liver cells [4,5]. In addition, four compounds (indole-3-carboxyaldehyde, wedelolactone, luteolin, apigenin) from plant extracts of *W. chinensis* showed prostate cancer prevention and therapy properties [6,7]. Moreover, the essential oils of *W. chinensis* showed antimicrobial, anti-inflammation, and antioxidant activities [8,9].

In comparison with the abundant pharmacological data and preclinical studies, information regarding plant tissue culture and regeneration of *W. chinensis* is rare. In vitro propagation of *W. chinensis* through nodal segments or axillary buds has previously been reported [1,10–12]. Moreover, three different explants (namely nodal segment, shoot apex, and leaf segment) of *W. chinensis* were grown on MS medium supplemented with

different combinations of cytokinins (6-benzyladenine (BA) and kinetin) and auxins (α-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA)); the nodal segment and shoot apex underwent direct organogenesis, giving rise to multiple shoot buds on MS medium supplemented with 3 mg/L BA and 0.5 mg/L IAA, whereas the leaf segment failed to proliferate in any of the media combinations [13]. To date, plant regeneration from leaf explants in *W. chinensis* has not been reported. In this study, optimal conditions of plant regeneration from leaf explants of *W. chinensis* were determined. In addition, we also established solid and liquid root cultures of *W. chinensis*.

2. Materials and Methods

2.1. Plant Material, Culture Conditions and Chemicals

Plant material of *Wedelia chinensis* (Osbeck) Merrill was kindly provided by Dr. Pei-Wen Hsiao, Academia Sinica, Taiwan. The authenticity of the plant species had previously been validated [6]. Fresh whole plants were grown and maintained in pots containing soil mixtures of Finnpeat (Kekkila, Finland) and King Root Gardening number 3 (King Root Gardening Company, Taiwan) in a 25 °C growth chamber under a cycle of 16 h illumination (100 μmol/m^2/s) and 8 h darkness. Preparation of in vitro-grown plantlets for propagation and plant regeneration from tissue explants in this species was recently reported [14]. Briefly, nodal segments were cut from the fresh plants, sterilized on a 125 mL flask containing 50 mL of 1% sodium hypochlorite and three drops of Tween-20 for 20 min, and thoroughly washed with sterile water. They were transferred onto MS medium [15] basal salt mixture (Duchefa Biochemia, Haarlem, Netherlands) supplemented with 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8. The culture was then incubated in a 25 °C growth chamber under a cycle of 16 h illumination (100 μmol/m^2/s) and 8 h darkness.

Gibberellic acid (GA_3), NAA, BA, kinetin, zeatin, IAA, indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and adenine were purchased from Sigma-Aldrich (St. Louis, MO, USA); zeatin, thidiazuron (TDZ), and *meta*-topolin were purchased from Duchefa Biochemia (Haarlem, Netherlands).

2.2. Establishment of Solid and Liquid Root Cultures

For solid root culture, leaves from in vitro-grown plantlets of *W. chinensis* were excised, cut into small pieces, and cultured on MS medium including vitamins (Duchefa Biochemia, Haarlem, Netherlands), 3 mg/L NAA, 3% sucrose, and 0.8% agar. The pH of the medium was adjusted to 5.8. The cultures were incubated for 1 month in a 25 °C growth chamber with a 16 h illumination photoperiod. For liquid root culture, the induced roots (0.5 g) were cut from above solid plates and transferred onto MS medium including vitamins, 3 mg/L NAA, and 3% sucrose. To prepare the control, 0.5 g wild-type roots were cut from solid plates and transferred onto MS medium including vitamins and 3% sucrose. The pH of the liquid medium was adjusted to 5.8. The culture was incubated for 1 month in a 25 °C shaker at 50 rpm in the dark, and root fresh weight was measured.

2.3. Shoot Regeneration from Mature Leaf and Stem Explants of In Vitro-Grown Plants

Mature leaf and stem explants from in vitro-grown plantlets of *W. chinensis* were excised, trimmed into small pieces and cultured on MS medium including vitamins, 3% sucrose, 0.8% agar supplemented with different kinds and different concentrations of cytokinins (including BA, kinetin, TDZ, *meta*-topolin) and auxins (including NAA, IAA, IBA, 2,4-D), alone or in combination, as follows: 2–4 mg/L BA; 2–4 mg/L IBA; 3 mg/L BA, 0.5 mg/L IBA; 3 mg/L BA, 0.5 mg/L NAA; 3 mg/L zeatin, 0.5 mg/L NAA; 0.1 mg/L TDZ, 0.5 mg/L NAA; 0.5 mg/L TDZ, 0.5 mg/L NAA; 3 mg/L zeatin; 3 mg/L zeatin, 0.2 mg/L NAA; 1 mg/L TDZ; 1 mg/L TDZ, 0.2 mg/L NAA; 3 mg/L BA, 0.1 mg/L NAA; 3 mg/L zeatin, 0.1 mg/L NAA; 1 mg/L TDZ, 0.1 mg/L NAA; 2 mg/L BA, 0.5 mg/L 2,4-D; 0.5 mg/L IAA; 0.5 mg/L TDZ, 0.2 mg/L NAA, 1 mg/L GA_3; 0.25 and 0.5 mg/L *meta*-topolin; 0.25 and 0.5 mg/L zeatin; 0.25 and 0.5 mg/L kinetin; 0.5 mg/L BA, 0.5 mg/L

kinetin, 3 mg/L zeatin, 0.2 mg/L NAA; 0.5 mg/L BA, 0.5 mg/L kinetin, 0.5 mg/L TDZ, 0.2 mg/L NAA. The pH of each medium was adjusted to 5.8. For each experiment, around 10 explants were incubated per Petri dish, and at least two replicates were carried out. The culture was then incubated for 1 month in a 25 °C growth chamber under a cycle of 16 h illumination (100 µmol/m^2/s) and 8 h darkness.

2.4. Plant Regeneration from Young Leaves of In Vitro-Grown Plants

Young leaves were excised from the in vitro-grown plants of *W. chinensis*. They were grown on culture medium (MS medium including vitamins, 0.5 mg/L NAA, 0.75 mg/L TDZ, 1 mg/L GA_3, 3.75 mg/L adenine, 3% sucrose, and 0.8% agar at pH 5.8) for 2 months in a 25 °C growth chamber with a 16 h illumination photoperiod. For each experiment, at least 25 young leaf explants were incubated per Petri dish, and 4 replicates were carried out. Regenerated shoots were excised and transferred onto MS medium basal salt mixture, 3% sucrose, 0.8% agar, and pH 5.8. The culture was further incubated in the same 25 °C growth chamber with a 16 h illumination photoperiod. The rooted plantlets were then transferred into soil and grown in a greenhouse.

3. Results

3.1. Establishment of Root Cultures from Leaf Explants

The root is an important organ for the synthesis and storage of certain primary and secondary metabolites, including for the accumulation of starch, proteins, and a wide diversity of phytochemicals [16]. Thus, we wanted to evaluate the biomass production of roots from *W. chinensis* by using a root culture technique. As shown in Figure 1a, abundant roots were induced in MS medium including vitamins supplemented with 3 mg/L NAA. However, no shoots were regenerated from the solid plates. Subsequently, roots (0.5 g fresh weight) were excised, transferred into liquid MS medium including vitamins supplemented with 3 mg/L NAA, and the cultures were incubated on a shaker (50 rpm) for 1 month in the dark. Higher root biomass of 1.76 ± 0.19 g fresh weight was obtained from five experiments (1.72, 2.04, 1.79, 1.52, and 1.71 g fresh weight) as compared to 1.24 g fresh weight of control (MS medium including vitamins without adding NAA). Due to contamination, only one replicate of control was available for the root culture data. Typical liquid culture is shown in Figure 1b. Thus, we concluded that 3 mg/L NAA is effective and sufficient for solid and liquid cultures in *W. chinensis*.

3.2. Evaluation of Shoot Regeneration from Leaf and Stem Explants

Although abundant roots were induced in MS medium including vitamins supplemented with 3 mg/L NAA, no shoots were observed. For direct morphogenesis, direct shoot formation is a critical step in plant regeneration. Those adventitious shoots from tissue explants had to be cut and transferred into rooting medium, and then complete regenerated plantlets with shoots and roots can be obtained [17]. It is well known that auxins (such as NAA, IBA, 2,4-D, etc.) can promote root formation, whereas cytokinins (such as BA, kinetin, zeatin, TDZ, *meta*-topolin, etc.) can promote shoot formation [18]. To promote shoot and plant regeneration, mature leaf and stem explants from in vitro-grown plantlets of *W. chinensis* were used, and media contained different kinds and different concentrations of plant growth regulators (PRGs), as described in the "Materials and Methods" section. No shoots were induced in these experiments. For example, no callus or shoot formation was observed from leaf and stem explants cultured in MS medium including vitamins supplemented with 2, 3, or 4 mg/L BA (Figure 2). BA, belonging to cytokinin, is the most common PGR for induction of adventitious shoots. In comparison with BA, abundant roots were induced from leaf explants which were cultured in MS medium including vitamins supplemented with 3–5 mg/L IBA (Figure 3a–c). IBA, belonging to auxin, is a PGR for induction of root formation. However, no calli or roots were observed in stem explants in the presence of 3–5 mg/L IBA (Figure 3d–f).

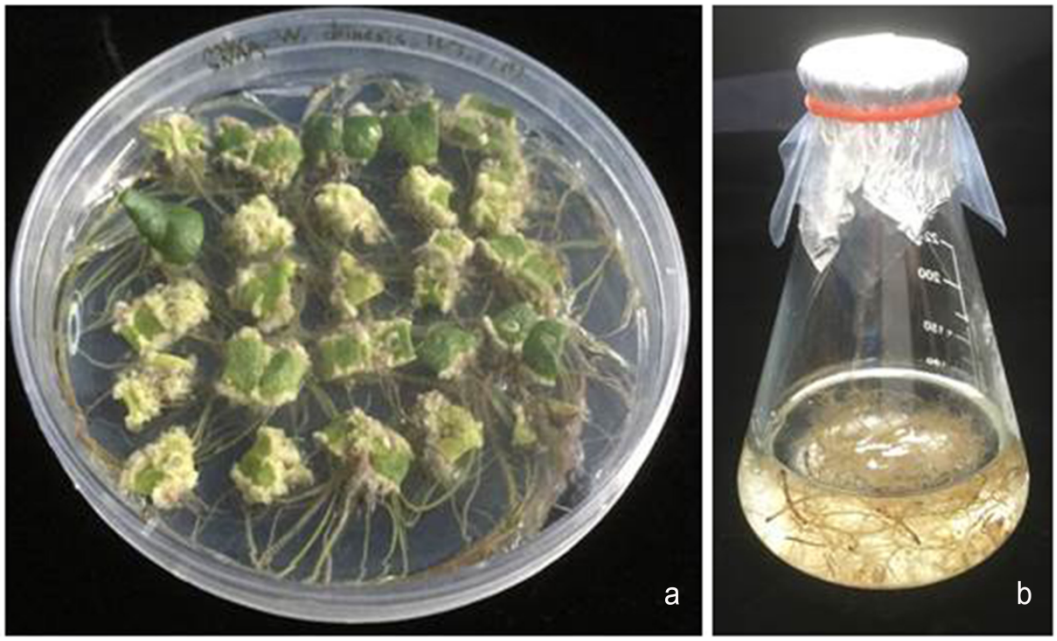

Figure 1. Solid root culture (**a**) and liquid root culture (**b**) of *Wedelia chinensis*. (**a**) Leaf segments from in vitro plantlets were excised and incubated on MS medium supplemented with 3 mg/L NAA, 3% sucrose, and 0.8% agar at pH 5.8 for 1 month in a 25 °C growth chamber with a 16 h illumination photoperiod. (**b**) For liquid root culture, 0.5 g roots were cut from leaf fragments cultured in solid medium plates and transferred in the medium without adding agar. The culture was incubated for 1 month in a 25 °C shaker at 50 rpm in the dark.

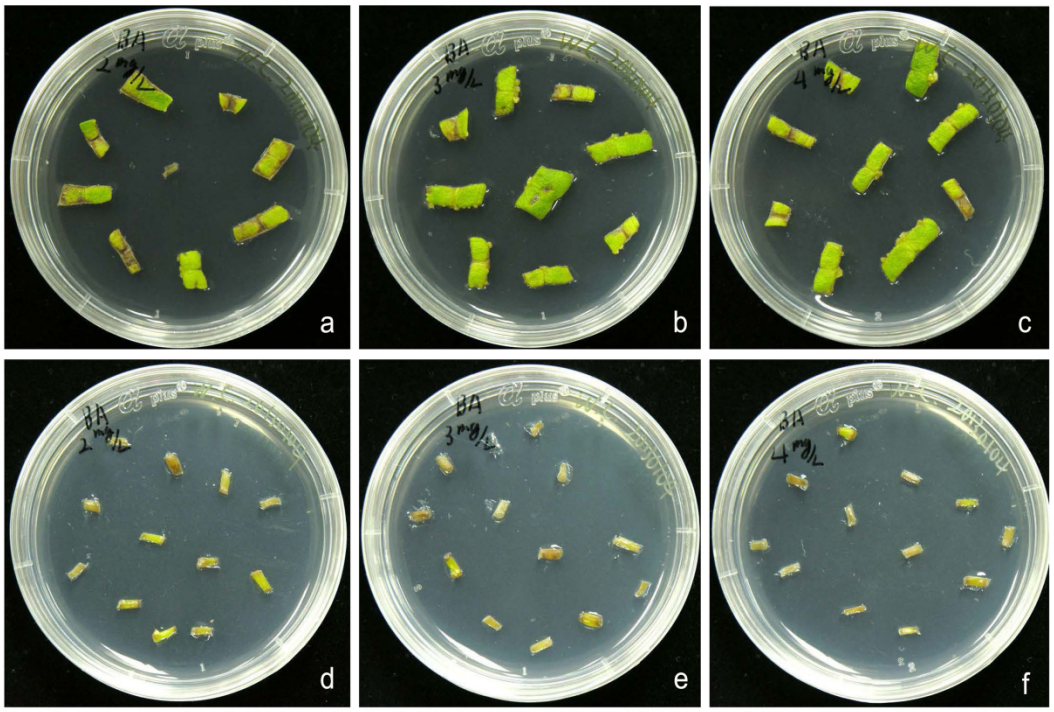

Figure 2. Mature leaf segments (**a**–**c**) or stem segments (**d**–**f**) grown on MS medium supplemented with 2 mg/L BA (**a**,**d**), 3 mg/L BA (**b**,**e**), or 4 mg/L BA (**c**,**f**) for 1 month in a 25 °C growth chamber.

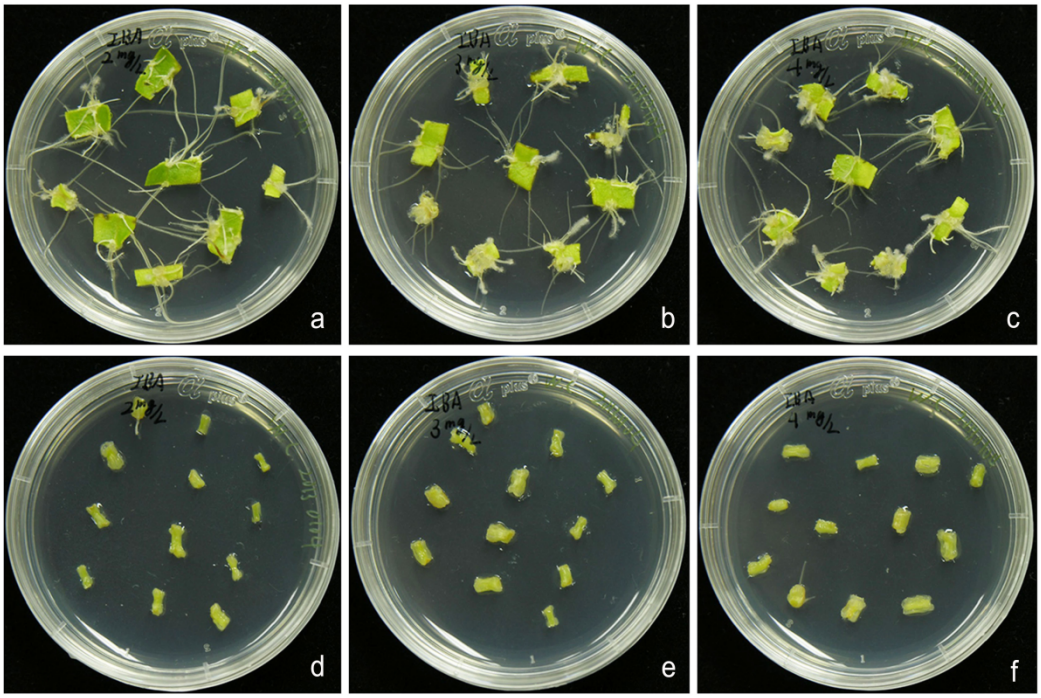

Figure 3. Mature leaf segments (**a**–**c**) or stem segments (**d**–**f**) grown on MS medium supplemented with 2 mg/L IBA (**a**,**d**), 3 mg/L IBA (**b**,**e**), or 4 mg/L IBA (**c**,**f**) for 1 month in a 25 °C growth chamber.

3.3. Plant Regeneration from Young Leaf Explants

After many experiments, we realized that the stem of *W. chinensis* is a tenacious tissue and does not respond to the PGRs under examination. Thus, we excluded stem explants in further experiments. Another crucial parameter for successful plant regeneration is the age of the tissue (leaf) explants. Shoot regeneration was not observed under any of the PGRs we examined from explants of mature leaves until the youngest top two leaves were used. As shown in Table 1, calli were observed in all explants derived from the youngest top two leaves, and the average percentage of shoot regeneration was 23 ± 5.20% from three independent experiments (Table 1). Then, several shoots were excised, transferred onto MS basal medium (MS medium basal salt mixture, 3% sucrose, 0.8% agar, pH 5.8), and cultured in a growth chamber for 1 to 2 months. Roots were easily induced. Finally, plantlets were transferred into soil, and all grew healthily in a greenhouse. No morphological variation was observed between the regenerated plantlets and the donor wild-type plants. Schematic representation of in vitro regeneration from leaf explants of *W. chinensis* is shown in Figure 4.

Table 1. Percentages of callus regeneration and shoot regeneration from young leaf explants of *Wedelia chinensis*.

Experiment	No. of Leaf Explants	No. of Explants Containing Calli (Percentage of Callus Regeneration)	No. of Explants Containing Shoots (Percentage of Shoot Regeneration)
1	125	125 (100%)	25 (20%)
2	100	100 (100%)	20 (20%)
3	109	100 (92%)	32 (29%)

Youngest top two leaves were cut into small pieces, incubated on culture medium for 2 months, and numbers of explants containing calli and shoots were independently scored.

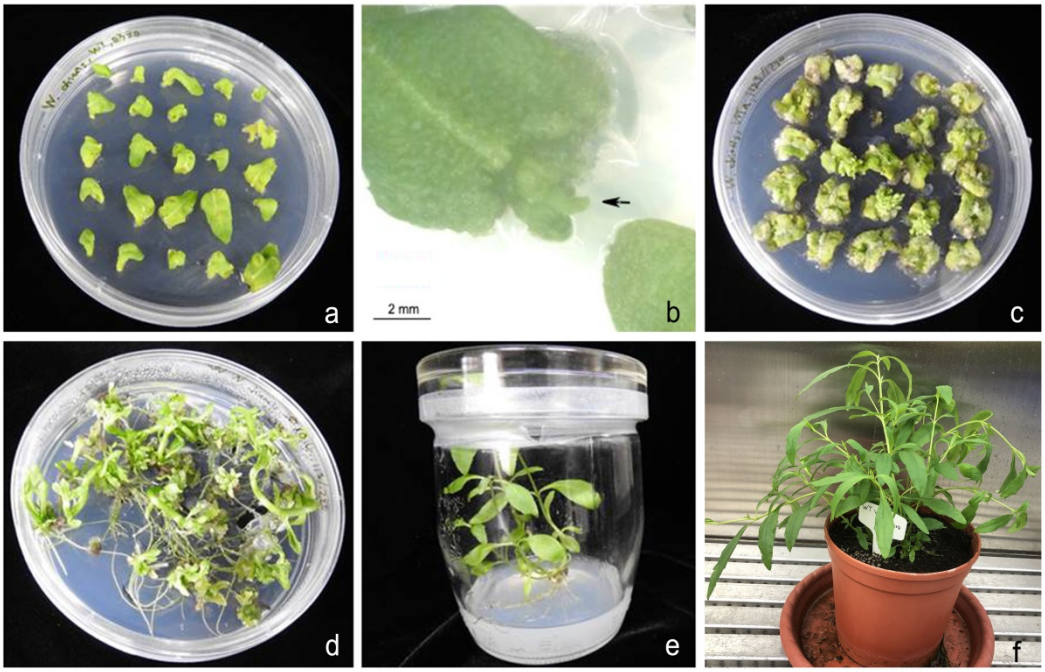

Figure 4. Plant regeneration from young leaf segments of the medicinal herb *Wedelia chinensis*. (**a**) Appearance after about 1 week of incubation on culture medium. (**b**) Under a light microscope, a callus (as indicated by the arrow) was observed at the edge of the young leaf segment after 1 week of incubation. (**c**) Shoots were observed after 1 month of incubation. (**d**) Roots were observed after 2 months of incubation. (**e**) Regenerated shoots were excised and transferred to the culture medium. Roots were observed after 1 to 2 months of incubation. (**f**) Plantlets were transferred onto soil and grown in a greenhouse.

4. Discussion

In this study, we established optimal conditions for plant regeneration from young leaf explants of the medicinal herb *W. chinensis*. To the best of our knowledge, plant regeneration from leaf explants of *W. chinensis* has not been previously reported. Obtaining in vitro plants of this species from leaf explants will be useful for propagation as well as genetic transformation of genes of interest into *W. chinensis*. Most transformation studies are based on the ability to attain plant regeneration from tissue explants; thus, development of a suitable regeneration system from leaf explants is a prerequisite for genetic transformation in *W. chinensis*.

It is well recognized that induction of shoot/root formation (especially shoot formation) from tissue explants by PGR is genotype dependent and tissue specific. Previously, we established plant regeneration and stable transformation from the floricultural plant *Cleome spinosa* and found that BA (0.5, 1.0, 2.0 mg/L) alone was sufficient to induce shoot regeneration from leaf explants; moreover, the percentage of shoot induction from hypocotyl, cotyledon, and leaf explants at the same concentration of BA showed a significant difference [19]. We tried different kinds and different concentrations of PGRs, alone or in combination; however, no regenerated shoots were observed in the stem or mature leaf from *W. chinensis* (Figures 2 and 3). We concluded that stems and mature leaves of *W. chinensis* are not suitable materials for plant regeneration and, therefore, we used only the youngest two leaves in the regeneration experiment (Figure 4). This is consistent with the findings that leaf segments from *W. chinensis* failed to proliferate in any of the media combinations under examination [13].

Organ cultures such as callus culture, shoot culture, suspension culture, root culture, etc., have been explored for the production of secondary metabolites for decades. It has been reported that more alkaloids (ajmalicine and catharanthine) accumulate in root culture as compared to shoot, callus, and leaf cultures in the periwinkle *Catharanthus roseus* [20]. Similarly, root culture produces a greater amount of tropane alkaloids (hyoscyamine and

scopolamine) as compared to suspension culture [21]. In *W. chinensis* (Osbeck) Merrill is useful for tattooing and dyeing gray hair; in addition, the root can be made into a powder and used as a black dye with salts of iron [1,11]. In this study, we established solid and liquid root cultures of this plant species (Figure 1) and may further extend the application of root culture.

In this study, we found that plant regeneration can be achieved in MS medium including vitamins supplemented with 0.5 mg/L NAA, 0.75 mg/L TDZ, 1 mg/L GA_3, and 3.75 mg/L adenine. NAA is an auxin, and at a higher concentration, NAA alone promotes root formation on the cutting edges of explants; however, at a low concentration (such as 0.5 mg/L) used together with cytokinin (such as TDZ), it also promotes callus initiation and adventitious shoot formation [18]. Previously, we also used 0.5 mg/L NAA in induction of polyploidy in *W. chinensis* [14]. To date, there are around 136 known members of the plant hormone class the gibberellins (GAs) [22]. The gibberellin most commonly used in plant tissue culture is GA_3. GAs are involved in a wide range of developmental responses, including promotion of elongation in stems and leaves, due in part to activation of the intercalary meristem. Another important role of GAs is the induction of hydrolytic enzymes such as α-amylase and protease in the seeds of cereals, hence facilitating endosperm mobilization. Other roles of GAs in some plants include promotion of seed germination, bolting of rosette plants, fruit development, and control of juvenility [23]. Adenine can be used as a plant cell culture additive. Adenine sulfate at a higher concentration (e.g., 25 mg/L) in the culture medium improves the frequency of multiple shoot production and allows recovery of the leaves from chlorotic symptoms according to the manufacturer's instruction (Sigma-Aldrich, St. Louis, MO, USA), and its applications in growth and development of shoot regeneration and inhibition of shoot tip necrosis have been reported [24,25]. Here, we observed that the addition of adenine sulfate at 3.75 mg/L can improve browning and shoot regeneration from leaf explants of *W. chinensis*. We also found that the most important factor for successful plant regeneration in *W. chinensis* is to use the youngest top two leaves as tissue explants.

Author Contributions: K.-Y.T. conceived and designed research. Y.-T.T. conducted experiments. K.-Y.T. and Y.-T.T. analyzed data. K.-Y.T. wrote the draft and the final version. All authors have read and agreed to the published version of the manuscript.

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

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzyladenine
GA_3	gibberellin A_3
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MS medium	Murashige and Skoog (1962) medium
NAA	α-naphthaleneacetic acid
PGR	plant growth regulator
TDZ	thidiazuron

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