In vitro rejuvenation of nodal segment explants for clonal propagation of Rauvolfia serpentina (L) Benth ex Kurz

Tasiu Isah

taasmore@yahoo.co.uk

Jamia Hamdard https://orcid.org/0000-0002-2112-530X

Nadia Zafar

Hamdard University New Delhi

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Abstract

Rauvolfia serpentina (L) Benth ex Kurz is an endangered medicinal woody species, widely distributed in Asia and used in several traditional medicine systems. Application of *in vitro* clonal propagation offers alternative strategies for biomass production useful in the production of pharmaceuticals but, difficulty in explant selection and low response to clonal production are impediment to the success. The present study evaluated efficiency of *in vitro* rejuvenation of nodal segment explants derived from basal offshoots and terminal buds collected across growth seasons and effect of serial subcultures on shoot morphogenesis in *R. serpentina*. Effect of culture medium strength (quarter, half and full strength MS) on shoot morphogenesis and proliferation through four (4) subcultures were also evaluated. Of the PGRs tested, BAP was more efficient over Kin and TDZ, and addition of NAA (0.5 mg L^{-1}) to the PGRs promoted shoot morphogenesis. Rhizogenesis was achieved using half basal MS medium added with IBA, NAA and IAA with IBA been the most efficient over other auxins tested. However, lower concentration of the IBA showed most appropriate results on good root differentiation. As a result, IBA has been the most efficient over other auxins tested but, lower concentration is the most appropriate for good root differentiation. Differential accumulation of pigment molecules and cellular osmolytes in response to the culture condition were evaluated in the dark-green and pale-green leaf morpho-types observed in the shoot cultures. Results of the present experiment suggests that explants collection season and PGRs influenced *in vitro* rejuvenation of nodal segment explants through physiological and biochemical changes essential for shoot morphogenesis.

Introduction

Plant cell, tissue and organ culture (PCTOC) techniques offer practical approach for the conservation of plant genetic resources and production of plant secondary metabolites (PSM) that are used in drugs production by the pharmaceutical industries (Isah 2015, 2016a). Its application is regarded an alternative strategy for the large-scale production of biomass and PSM at industrial scale, so as to mitigate over exploitation of field growing woody plants (Guan et al. 2016; Isah et al. 2018). Among their characteristics is the long juveline period during which the rate of vegetative growth becomes rapid, and reversal of the maturity through physiological molecular expression modification enables recovery of rooting ability through rejuvenation for micropropagation (Hackett 1985; Poethig 2013; Isah 2016b). During early years of PCTOC in the woody species, biological basis of phase change and its stability were not well understood but, believed to be associated with cellular division and influence of endogenous hormones, the cellular isolation and interactions that occurs at physiological and anatomical background levels of juvenile and mature explants (Hackett 1985). This, led to the development of strategies for rejuvenating mature plant material through prunning, etiolation, repeated grafting and successive subculture (Wendling et al. 2014; Vidoy-Mercado et al. 2021) with cellular and subcellular anatomical features regarded as the determinants of explant rejuvenation efficiency (Bonga 1987, 2017).

Rauvolfia serpentina (L) Benth ex Kurz (Apocynaceae) is a medicinal perrennial woody shrub and rich in the production of terpenoid indole alkaloids (ajmaline, ajmalicine, deserpidine, recinnamine, reserpine,

serpentine and yohimbine) in most of its parts (Siddiqui 1939; Bahuguna et al. 2011; Mukherjee et al. 2019). Because of the low propagation rate of its seeds and using vegetative methods, collection of the wild populations has resulted in its threatened status with extinction possibilities in the near future, and currently classified endangered due to increasing decline in its populations (Susila et al. 2013; Ghate et al. 2019). Propagation of *R. serpentina* plants through the seeds is highly challenging because of their low germination frequency attributed to many phytochemicals produced by the seeds, and application of pre-germination treatment is less efficient in stimulating the germination (Mitra 1976). Application of PCTOC techniques, determined by explant choice and preparation, culture medium and plant growth regulators (PGRs) supplemented, culture conditions and micro environment of incubation (Bonga 2017) for the establishment of shoot morphogenesis, hairy root cultures and genetic transformation offer alternative biotechnnological strategies for biomass and PSMs (alkaloids) production in *R. serpentina*, so as to mitigate overexploitation of its field growing population (Rani et al. 2014; Gantait et al. 2017; Mukherjee et al. 2019, 2020). Explants such as shoot-tip and axillary meristem, leaf and seed, nodal and internodal segments, hairy roots and encapsulated plants had been used in the *in vitro* clonal propagation of the species, and for the production of its important alkaloids (Senapati et al. 2013; Gantait et al. 2017; Mukherjee et al. 2019). However, nodal segment explant is regarded as the most suitable for multiple shoot morphogenesis and large-scale clonal production, possibly due to inherent presence of multiple axillary buds or their meristems in the nodal segment explants that facilitates axillary or *de novo* apical shoot bud initiation, although not yet established at the molecular levels. In the present experiment, nodal segment explants from the basal offshoot and terminal buds collected in two (2) seasons were assessed for morphogenic response studies through *in vitro* rejuvenation and plant recovery in *R. serpentina*. Effectiveness of the culture medium strength on shoot morphogenesis and differential accumulation of pigment molecules in two (2) observed morphometric leaves of the micro shoot cultures were also analyzed.

Materials And Methods

Explants collection and preparation

Physiologically healthy and tender nodal segment explants from the basal offshoot and terminal buds of *R. serpentina* plants growing in the herbal garden of Botany Department, Hamdard University New Delhi, India were regularly collected during the months of May – June (Summer) and December – February (Winter) periods in 2015. The explants source-selection was principally based on sanitary and good physiological state of field growing plants, and at later stages, from elongated shoot cultures derived from the various explants. Field-collected explants were washed thoroughly under running tap water for 3–5 mins before transferred to laminar airflowhood chamber for surface sterilization. The explants were stirred in 1.0% Tween – 20 solution for 25 mins, and then rinsed with distilled water to remove traces of the detergent. They were then treated with ethanol (70%) for 1–2 mins, rinsed with distilled water, followed by treatment with $HgCl_2$ (0.1%) for about 1 min. Explants were again rinsed thrice with sterile distilled water to remove traces of the $HgCl_2$ prior to cultivation on solid medium. The explants (0.5–1

cm) were cut at the basal end before cultured on solid Murashige and Skoog (1962) medium (Full, half and quarter strength) that contained sucrose (3.0% w/v), Agar (0.8% w/v) and amended with cytokinins viz BAP, TDZ and Kin (0.5, 1.0, 1.5 2.0, 2.5 and 3.0 mg L^{-1}) and later in combination with lower levels of NAA (0.5 mg L^{-1}). Media pH were adjusted to 5.7, dispensed (about 5–10 ml) into 25 x 150 mm test tubes (Borosil, India) and sealed with non-absorbent cotton plugs before autoclaved at 10321.35 **pa** at 121**°C** for 15 mins. All cultures were kept at 24 ± 1 **°C** under a 16 h photoperiod that was provided by cool white fluorescent lamps of 100µ mol m^{-2} s^{-1} photon flux density (Phillips, India) and periodically, morphogenic responses visually observed. Upon axillary buds sprouting, shoots were excised from the nodal segment (basal offshoot and terminal bud) and subcultured after 3–4 weeks culture on solid MS medium (quarter, half basal and full strength) without (control) and added with PGRs for up to 4 subcultures prior to final data collection for elongation, rhizogenesis and analysis. The number of shoot buds produced per subcultured shoot was taken as the coefficient of shoot proliferation after 4 subcultures. Data on the morphogenic response was also collected after 5 weeks culture of micro shoots during the 4th subculture as the overall number of morphogenic shoot structures observed per cultured nodal segment without regards to its type. Percentage of shoot morphogenesis and rhizogenesis were expressed as:

$$\textbf{Percentage response} = \frac{\text{Total number of respondent shoot cultures or rooting}}{\text{Total number of replicates}} \times 100$$

Rooting of micro shoot

Well developed shoots (after 4 subcultures) were used for the rooting experiment; for rhizogenesis of the micro shoots, proliferated shoot cultures were selected, excised at the basal end, cultivated on solid root induction and proliferation half basal MS medium supplemented with IBA, NAA or IAA concentrations (0.5, 1.0, 2.0 and 3.0 mg L^{-1}) and within few days, root differentiation observed. Plantlets were allowed to develop good root system by incubation for 3–4 weeks on the half basal solid MS medium augmented with the auxins. Data on the average number of roots formed per micro shoot were recorded after 3 weeks cultivation, and well-developed plantlets aclimatized in small rubber pots containing mixtures of soilrite and soil (1:1). Recovered plantlets were covered with polyethene bags to maintain high relative humidity and reduce excessive transpiration that may reduce frequency of plants recovery, and aclimatized plants, under standard fluctuating summer period temperatures, transferred to herbal garden field conditions (explant source-environment).

Leaves pigments and cellular osmolytes contents analysis

Chlorophyll (Chl) a and b, total Chl and carotenoids (Cars) content in the leaves of dark green (DG) and pale green (PG) micro shoot cultures were evaluated and their concentration determined according to earlier reports (Arnon, 1949; Wellburn, 1994). The leaves were harvested from the cultures and pigment content extracted using 80% cold acetone under dark condition. About 200 mg fresh weight (FW) of the various leaves samples (DG and PG) were separately taken, mixed with acetone (cold) and macerated

using mortar and pestle. Extracts were then transferred into a 2 ml-capacity centrifuge tubes, and then centrifuged at 10, 000 rpm for 10 mins at 4^{o}C. The obtained pellets were washed using 80% cold acetone until the coloration completely disappeared. Supernatants obtained were then collected (in test tubes) and diluted up to 5 ml, followed by samples readings taking using spectrophotometer (Shimadzu, Japan) at 662 nm in the case of Chl a, 644 nm for Chl b, 662 for Chl a + b and 440 nm for the Cars.

Chlorophyll **a** = 9.784·A662–0.990·**A644**

Chlorophyll **b** = 21.426·A644–4.650·**A662**

Chlorophyll **a + b** = 5.134·**A662** + 20.436·**A644**

Carotenoids = 4.695·**A440–0**.268·(**a + b**);

Following calculation of the concentrations, amounts of the pigments g^{-1} of leaves FW were calculated using the formula:

C = C_1 Vr**/**m where
C = content of pigment molecules (mg g^{-1}) of fresh leaves weight

C1 = concentration of calculated pigment molecule by the previous formula (mg L^{-1});

v = starting volume of extracts (mL);

r = dilution;

m = weighed fresh plant leaves (g).

Glycinebetaine content of the DG and PG leaves were measured using the periodic calorimetric method of Grieve and Grattan (1983) as modified by Lokhande et al. (2010). Various fresh leaves – separately (500 mg) were grounded in the liquid nitrogen and fine powder obtained mechanically shaken in deionized water (20 ml) for about 16 h on an orbital shaker at 25^{o}C. The samples were then filtered and the filtrate obtained (500 µl) diluted (1:1) using 2N H_2SO_4. Extracts were then cooled in an ice block for about 1 h before mixed with I_2-KI (200 µl) reagent (Iodine 15.7% - Potassium iodide 20%). The tubes were then gently mixed prior to storage at 4^{o}C for 16 h and centrifugation at 10, 000 rpm for 15 mins at 0^{o}C. Periodic crystals formed were dissolved in 1, 2-dichloromethane (9.0 ml) and after 2 h, absorbance measured with spectrophotometer at 365 nm. Contents of the glycinebetaine were evaluated using standard curve prepared with a glycinebetaine standard, and expressed in µg g^{-1} FW.

Proline contents were analyzed based on earlier report (Bates et al. 1973). In brief, 500 mg of the various fresh leaves tissue samples (DG and PG) were homogenized in an aqueous 5 -sulfosalicylic acid (3% v/v) and the residue centrifuged at 10, 000 g for 10 mins at 4^{o}C. Supernatant (2 ml) obtained was reacted with acid ninhydrin (2 ml) and glacial acetic acid (2 ml) through incubation at 100^{o}C in a hot water bath

for about 1 h and reaction terminated in an ice bath, and allowed to cool to the room temperature. Reaction mixture was then extracted with toluene (4 ml) and vigorously stirred for 10–15 seconds. Chromophore-containing toluene was aspirated from aqueous phase, followed by warmed to room temperature and its absorbance measured at 520 nm using toluene as blank. Concentration of proline in the samples was determined from the standard curve drawn using L-proline as standard, and expressed in μg proline g^{-1} FW.

Total soluble sugars (TSS) of the leaves were determined using anthrone method (Watanabe et al. 2000) modified by Lokhande et al. (2010). In brief, various leaves (separate) samples (200 mg each) were homogenized in ice-chilled ethanol (80%) by using mortar and pestle. Centrifugation was used in preparation of the extracts (5, 000 g for 10 minutes at 4^{o}C) and final volume adjusted to 10 ml using 80% ethanol. The supernatant (1 ml) recovered was reacted with freshly prepared anthrone reagent (3 ml) by incubating the mixture in hot water bath for 10 mins at 100^{o}C. The reactions were then terminated by quick cooling in ice bath, then allowed to cool at room temperature, and absorbance of these mixtures measured at 620 nm. Standard curve was prepared using D-glucose for the estimation of TSS, and obtained results expressed in mg g^{-1} FW.

Statistical analysis

The experiments were arranged in a complete randomized block design. Data generated from triplicate experiments consisting of 8 replicates per treament (repeated twice) on shoot morphogenesis, elongation, number of leaves per micro shoot, rhizogenesis and pigment molecule content analysis from the DG and PG leaves of micro shoot cultures were analyzed using SPSS ver. 21 (USA). Results are presented as mean ± standard error of the replicated experiments. Significant differences between the treatments were assessed by analysis of variance (ANOVA) followed by a Tukey's range test assessment at probability levels of $p \leq 0.05$.

Results

In the present experiment, morphological and physio-biochemical changes associated with *in vitro* rejuvenation events were spectrophotometrically studied and through observations of mature nodal segment explants derived from the basal offshoot and terminal buds collected in the summer and winter periods and the initiated shoots. Shoot initiation from the mature explants, their proliferation, rooting response and survival at aclimatization stages also analyzed.

For initiation of the aseptic cultures, fifty (50) explants, each from the basal offshoot and terminal buds (25 for each collection season) of *R. serpentina* were collected and surface sterilized under similar exposure duration to the sterilants. Terminal buds, though having less degree of differentiation over the basal offshoot, showed minimal survival to exposure to the sterilizing agents, suggesting their effect on explants cells and tissues survival. This was reflected by the influence on the physiological state of explants which influenced degree of response to *in vitro* morphogenesis, that also reflected collection season of the explants (Fig. 1a–c; Fig. 2a). Analysis of the results of the hundred (100) explants used

showed significant difference between the explants across collection seasons with respect to efficiency of surface sterilization technique used and resultant shoot morphogenesis during the cultivation period (Fig. 2a). Explants collected in the winter period showed high frequency (%) of disinfection over the summer with 72.1 ± 0.17 for basal offshoot and 68.3 ± 0.23 with terminal buds when compared with the summer where 52.42 ± 0.24 and 47.15 ± 0.83 were respectively recorded, suggesting the influence of temperature of the two (2) collection seasons on microbial survival on explants surface and efficiency of the disinfection treatments to explants recovery.

In the case of shoot initiation, both basal offshoot and terminal buds showed good response to shoot bud differentiation but, in the overall, terminal bud explants showed higher rate of the morphogenic response through differentiation of thickened “bud-like” structures that further differentiated into shoot bud within 2–3 weeks cultivation. The response was higher and earlier on medium added with PGRs (above 1.0 mg L^{-1}) but, with prolonged cultivation through subcultures, was accompanied with basal callusing due to the possible influence of endogenous hormones in explants. Initiated shoot cultures were serially subcultured on fresh medium after 3 weeks cultivation for up to 4 subcultures, and after the subcultures, adventitious shoots formation were observed in most cultures (Fig. 1d–e), with profuse branching in high PGRs amended medium (above 1.0 mg L^{-1}). The number of shoot buds per explant increased with increase in the subcultures, and shoot buds emergence was more during early days of culture into fresh media; with elongation and branching of the micro shoots, adventitious shoot formation observed with some cases of callusing at the cut-end of micro shoots, while leaves acquired increased juvenile appearance with some flaccid characteristics (Fig. 1d–n and 2b–d). Adventitious shooting was more prevalent in shoots cultivated on solid medium supplemented with BAP and TDZ, especially higher levels of above 1.0 mg L^{-1} (Fig. 1e). Maximum of 5 shoots per explant were recorded after 4 subculture cycles, with adventitious shoot formation prevalent in cultures cultivated on medium amended with BAP and TDZ concentrations, especially their higher levels (Fig. 1e). It was observed that the number of shoot buds per nodal segment explant from the terminal bud was higher over the basal offshoot, especially in high cytokinin amended medias, and often showed bushy and profuse shoot buds induction. An average of 5.0 shoots were observed per nodal segment explants of the terminal bud during the 4th subculture while in the case of basal offshoots, it was about 2–3 per nodal segment in high cytokinin supplemented medias. However, lower concentration of the cytokinins were efficient in producing quality and slender shoots during proliferation: higher concentrations produced thick-bushy shoots that were short in the case of TDZ-supplemented medias (Fig. 1d–n and 2b–c). Further, supplementing the cytokinin-added medium with lower levels of NAA (0.5 mg L^{-1}) improved the morphogenic response throughout the subcultures: an increase in shoot length were observed during shoot proliferation but, was higher on BAP amended medium levels over that of Kin and TDZ. Similarly, morphogenesis of leaves through their size and number were accordingly associated with the PGRs supplemented in the cultivation medium. Maximum average number of leaves were recorded in BAP-supplemented medias over other PGRs tested, and was influenced by the cultivation medium strength. However, two (2) morphometric leaf types were observed –DG (Fig. 1k-l) and PG (Fig. 1m), with the leaves been less developed during the 1st and 2nd subcultures but, showed expansion and advancement in development with increase in the subcultures

(Fig. 1c–h). Maximum average number of leaves were recorded on media supplemented with BAP (2.0 mg L^{-1}) where an average of 33.2 ± 0.14 leaves were produced per micro shoot after four (4) subcultures and least with the Kin where 2.11 ± 0.17 leaves were produced in media added with 0.5 mg L^{-1} of the cytokinins (Fig. 2d).

For root morphogenesis, compared to IAA and NAA treatments, addition of IBA to half basal MS medium promoted the number of roots with a maximum average of 12.14 ± 0.24 roots on medium added with 1.0 mg L^{-1} IBA over the 9.71 ± 0.31 in 2.0 mg L^{-1} NAA supplemented medium and 5.26 ± 0.19 for 1.0 mg L^{-1} IAA, suggesting its role in rhizogenesis to be principally on the number of roots induced per micro shoot.

In the present experiment, the two morphometric leaf types observed were subjected to physiological-biochemical analysis of pigment molecules and cellular osmolytes; Chla content of the PG leaves was lower than that of the DG leaves with 0.27 ± 0.13 and 0.87 ± 0.18 mg g^{-1} FW respectively while Chlb content was higher in the PG leaves with 0.29 ± 0.16 mg g^{-1} FW over the DG ones that accumulated 0.176 ± 0.15 mg g^{-1} FW. In the case of total Chl content, DG leaves accumulated 1.20 ± 0.18 mg g^{-1} FW over the PG leaves that had lesser of 0.38 ± 0.14 mg g^{-1} FW. For Car content, it was higher in the DG leaves with an average of 0.48 ± 0.13 mg g^{-1} FW over the PG leaves that accumulated 0.36 ± 0.16 mg g^{-1} FW, suggesting that higher accumulation of Cars in DG leaves over the pale ones to be due to the possible superior adaptative response of leaves to excessive light irradiation of the *in vitro* culture. In the case of total soluble sugars (TSS), their synthesis was higher in PG leaves with 25.13 ± 0.24 mg g^{-1} FW over the 9.12 ± 0.17 mg g^{-1} FW in the DG leaves while glycinebetaine accumulation was relatively higher in PG leaves where 158.14 ± 0.21 mg g^{-1} FW were produced over the dark ones that had 118.12 ± 0.11 mg g^{-1} FW. For proline, its content was higher in the PG leaves with 153.19 ± 0.13 mg g^{-1} FW produced over the DG ones that had 103.45 ± 0.15 mg g^{-1} FW (Fig. 3b).

Discussions

Explants preparation and cultivation

One of the key steps in the *in vitro* clonal propagation of woody plants is appropriate and efficient explant selection and surface sterilization to avoid growing contaminants, i.e infection with bacteria and fungi in culture medias (Viss et al. 1991; Orlikowska et al. 2017). Keeping up with the principal experimental objective, basal offshoot and terminal bud explants were used in the present experiment, and sterilizing agent dosage and duration of treatment showed influence of collection season on efficiency of the surface sterilisation method used for removal of microbial contaminants from the summer and winter collected nodal segments, which also determined the efficiency of healthy explants recovery (Fig. 1a–c; Fig. 2a). This is because latent bacterial endophytes that may be present on cultured plant tissues could produce toxins that can cause tissue necrosis when present in sufficient amount but, supplementing antibiotics in the culture medium at proportionate levels is regarded an effective strategy for controlling contamination from plant systems (Reed and Tanprasert 1995; Reed et al. 1998; Javed et al. 2017). In the

present study, levels of contamination (percentage) recorded, in most cases bacterial, varied with explant collection season, which is similar to the earlier experience encountered in *Ginkgo biloba* L. *in vitro* cultures (Isah 2019a), especially when the plant material is collected from natural environment (Seth et al. 2007). One of the great challenges in the establishment of aseptic cultures from field-grown plants-collected explants is the contaminants present in explant surface (Viss et al. 1991). Thus, on the basis of plants growth season makes their removal difficult, with winter period been the most favourable over the summer because it enables them persist in tissues without showing element of contamination in cultures.

Because of the presence of buds near basal shoot zone, it is recommended as the best explant-source for *in vitro* rejuvenation experiments due to its juvenile characteristics (Bonga 1987). Although in some instances such explants show exudation of phenolic compounds into solid culture medium but, subsequent transfer to fresh media amended with similar composition could ameliorate the effect (Preece and Compton 1991). In the present study, browning effect was observed within two (2) weeks of explants cultivation. Basal offshoot showed earlier response to shoot morphogenesis over the terminal bud nodes, with early stage of their induction characterized by thickened shoot emergence, resembling adventitious buds from existing buds, possibly due to the higher levels of endogenous hormones in the explant (Khan et al. 2014). Generally, juvenile tissues are regarded to have higher sensitivity to PGRs because of their high auxin levels while in the case of cytokinin concentrations in mature explants, it increases with the maturation (Xiao et al. 2014; Zhang et al. 2020). Hence, *in vitro* rejuvenation inductive signal that results into shoot bud morphogenesis might be explained by disturbance in balanced distribution of endogenous hormones induced by PGRs amended in the culture medium of explants (Pinero et al. 2014). Thus, promoting active division of the quiescent parental cells that results in the initiation of stem-cell-like niche in explants. However, overall response was earlier with the terminal buds over the basal offshoot (Fig. 1a–c). Explant rejuvenation in trees is also associated with increase in levels of auxins, cytokinins and reductions in ABA levels, thus, promoting plant growth and development (Valdes et al. 2003). In most of the woody species, potentials for disinfection and morphogenesis from mature donor plant material is usually lower than that of juvenile, and in many instances, recalcitrance to *in vitro* culture may be encountered (Viss et al. 1991; Bonga et al. 2010). This is due to the loss of morphogenic potential that arises from tissue specialization, which is principally based on cellular function that reduces their plasticity imposed by cellular differentiation arising from genomic expression regulations (Bonga 2017; Ojolo et al. 2018).

Shoot morphogenesis

Healthy nodal segment explant is among the most prepared donor plant material for *in vitro* clonal propagation of woody plants, because of its pre-existing meristematic tissue that can be influenced to develop into shoot by PGRs supplemented in the culture medium (Viss et al. 1991). Levels of cytokinins in mature trees decreases with increase in maturation of explants, thereby, contribute in reducing cellular division and bud induction with promotory effect on plant tissue aging (Valdés et al. 2003). Hence, balance of endogenous and exogenous hormones and associated interactions determine shoot

morphogenesis in the *in vitro* cultures (Isah and Singh 2021). Serial micropropagation is regarded suitable alternative technique for rejuvenating adult trees, and levels of rejuvenation doesnt have effect on morphological features. However, progressive decline in explant memory to cell reprogramming occurs after introduction to the *in vitro* culture and with increase in subcultures (Beck et al. 1998). In a recent reported experiment, it was observed that explant collection season influenced *in vitro* morphogenic response using nodal segment explants (Isah 2019a). Such experience prompted assessment with *R. serpentina* for which explants collected during December – February showed low response to shoot bud induction when compared with that of May – July (Fig. 1a–c), with medium browning prevalent in explants collected in the months outside the stated range of the calendar year. Explants of 2–3 cm length were prepared and cultured on quarter, half basal and full strength solid MS medium amended with PGRs at varied concentration levels, and shoot "stock cultures" subcultured at 3–4 weeks intervals. Bud break from nodal segments were observed within 2–3 weeks of cultivation at varied degree, reflecting influence of the PGR types and concentration supplemented in the culture medium and explant used. This could be attributed to the more active vegetative growth of terminal buds over the basal offshoot explants during the donor plants growth season, which is similar to the experience encountered by Phulwaria et al. (2011) in *Salvadora persica* L. and Vibha et al. (2013) in *Dalbergia sissoo* (Roxb.) Kuntze. In culture medias supplemented with higher levels of the cytokinin concentrations (above 2.0 mg L^{-1}), basal callusing was prevalent with shoot cultures due to the possible strong influence of the amended cytokinins on apical dominance (Preece et al. 1991) in the species. The type of explant used for *in vitro* clonal propagation experiment influences efficiency of shoot morphogenesis, and intermediate and terminal position-derived buds are regarded as the most appropriate for use. Based on comparison across PGRs amended in the culture medias, shoot differentiation from terminal buds (2 wks) was earlier over the basal offshoot explants (3 wks) but, overall data was difficult to analyze as sufficient and independent data worthy of presentation. Hence, it was combined as overall effect of PGRs on shoot morphogenesis without expressing the differences in the two (2) nodal segment explants used in the experiment. The most responsive explant to shoot bud initiation/rejuvenation (data not shown) was those excised from terminal bud having buds near basal zone of field grown *R. serpentina* plants, possibly due to the prolonged retention of juvenile characteristics. This among many other reasons, can be explained by the lower sensitivity of mature plant tissues to cytokinins due to the possible binding of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) that targets miR156 to B-type ARRs (Zhang et al. 2015) that may be linked to explant rejuvenation and shift in the expression levels of *miR156* and *miR172* through accumulation of *miR156* (Heide 2019). From physiological point of view, it can also be attributed to differential and high levels of auxins in the terminal buds and increased levels of cytokinins in different seasons within cells, which is usually promotory to growth and development (Valdés et al. 2003). The number of shoot buds per nodal segment explant from the terminal bud was also higher over the basal offshoot, especially in high cytokinin amended medias, and often showed bushy and profuse shoot buds induction (Fig. 1d–n and 2b–c). High shoot initiation response achieved within the explants collection months could also be explained by active vegetative growth of *R. serpentina* plants during the period, which is similar to the experience encountered by Phulwaria et al. (2011) and Vibha et al. (2013). Initiated shoot cultures were serially subcultured on fresh medium after 3 weeks cultivation for up to 4

subcultures, and after the subcultures, adventitious shoots formation were observed in most cultures (Fig. 1d–e), with profuse branching in high PGRs amended medias (above 1.0 mg L^{-1}). The number of shoot buds per explant increased with increase in the subcultures, and shoot buds emergence was more during early days of culture into fresh media; with elongation and branching of the micro shoots, adventitious shoot formation observed with some cases of callusing at the cut-end of micro shoots while leaves acquired increased juvenile appearance with some flaccid characteristics (Fig. 1d–n and 2b–d). Adventitious shooting was more prevalent in shoots cultivated on solid medium supplemented with BAP and TDZ, especially higher levels of above 1.0 mg L^{-1} (Fig. 1e). In the present experiment, substantial variation on number of shoots per explant and their length was observed with the subculture cycle: with increase in the subculture cycle, the number of micro shoots produced per explant increased up to the third subculture cycle but, after wards showed negligible or non increase in the subsequent subcultures (personal observation). Maximum number of shoots and shoot length were observed with cytokinins (BAP and Kin) and TDZ-supplemented half basal MS over the quarter and full strength medium, and of the concentrations tested, BAP (2.0 mg L^{-1}) was more efficient over Kin and TDZ where 1.0 mg L^{-1} was the most effective in inducing the shoots (Fig. 2b–d) respectively. However most of the TDZ-induced micro shoots were lesser in height but bushy in appearance when compared with the slender shoots obtained with Kin-supplemented medium (Fig. 1d–n). This can be explained by the superior ability of plant tissue in absorbing and metabolizing BAP than other cytokinins tested (Zhang et al. 2010) and small less complex newly-formed micro shoot may result in vigorous and less lignified shoots with higher capacity for rhizogenesis (Wendling et al. 2014). Low salt concentration in the culture medium has been reported efficient in shoot morphogenesis and proliferation in many woody species that includes *Azadirachta indica* A.Juss. (Chaturvedi et al. 2004), *Terminalia arjuna* (Roxb.) Wight & Arn. (Pandey et al. 2006), T. *bellerica* (Gaertn.) Roxb. (Dangi et al. 2014) and in *Chonemorpha fragrans* (Moon) Alston (Isah and Umar 2018).

To further assess possible synergistic effect of auxin with cytokinins on the efficiency to shoot morphogenesis, the most efficient concentrations of BAP, Kin and TDZ were supplemented with NAA (.05 mg L^{-1}), i.e BAP (2.0 mg L^{-1}), TDZ (1.0 mg L^{-1}) and Kin (1.0 mg L^{-1}). Among the advantages of adding auxins to cytokinin-supplemented medium was reported to be the neutralizing effect it imposes in high cytokinins concentration, particularly during shoot elongation (Hu and Wang 1983). In the present experiment, maximum shoot morphogenesis was achieved with BAP + NAA (2.0 + 0.5 mg L^{-1}) where an average of 48.16 ± 0.15 shoots were produced per nodal segment explant after four (4) subcultures (Fig. 2e). However, lower concentration of the PGRs produced more healthy-looking shoots (Fig. 1j) and the number of shoots per explant was lower (2.31 ± 0.17). In the case of TDZ-supplemented cultures, majority of the shoot induction was acccompanied with some basal callusing, which was more prevalent when NAA (0.5 mg L^{-1}) was added to the TDZ-supplemented cultures (Fig. 1k). Leaves of most of the produced micro shoots through the rejuvenation showed extensive thickness and regreening (although two morpho-types were observed), suggesting their high photosynthetic activity due to possible relative high Chl contents. Takeno et al. (1992) suggested that such changes are induced by Kinetin, IAA or BAP through the roles they play in promoting cell growth. In Strawberry tissue cultures, BAP and GA_3 promoted

juvenile characteristics through the production of runners and monofoliate leaves but, with net increase in their diffusion resistance and reduction in net photosynthetic rates (Mohamed et al. 1991). Similar regulatory system was reported in rejuvenated apple trees for which due to subcultures, the levels of IAA in leaf tissues increased, leading to significant decrease in ABA contents (Xiao et al. 2014). In the case of *Nicotiana tabacum* L. rejuvenation, transient increase in cytokinin riboside and isopentenyladenosine was observed after 24 hrs cultivation but, levels of GA_3 increased only after 72 hrs culture (Jayaraman et al. 2016).

Shoot elongation

In the *in vitro* rejuvenation experiment in woody plant(s) tissue cultures, the (1) morphological markers of cellular differentiation through the number of leaves, their shape and size, branching pattern of shoots, and adventitious root formation (2) changes in physiological marker traits in the form of pattern of photosynthetic capacity and resistance to stress are of paramount importance (Kerstetter and Poethig 1998). Number of subcultures of micro shoots is also associated with physiological changes in the cultivated shoots, and successive subcultures rejuvenates plant material accompanied with decline in regeneration capacity after long-term subcultures (Su 2000). In the present experiment, increased shoot proliferation/elongation was achieved when micro shoots were excised and repeatedly subcultured onto fresh solid medium of similar composition, and with increase in passages during shoot proliferation, increase in number of shoot clumps per shoot to about 5 per initial shoot bud were observed (Fig. 2b–d), possibly due to rejuvenation of plant materials over the subcultures (Phulwaria et al. 2011; Goyal et al. 2012). However, basal callusing had to be removed during the successive subcultures (Fig. 1e). In earlier study by Alatar (2015) increase in shoot morphogenesis and proliferation parameters during each subculture cyle of micro shoot were reported in *R. serpentina*. Further, age-related physiological changes that increase shoot size may lead to decrease in vigor due to physiological aging (Hackett 1985) with number of subcultures related to physiological changes associated with vigor of rejuvenation events in the cultivated shoot cultures, and capacity for regeneration declines after long-term subcultures (Su 2000). Based on the physiological stage of explants, juvenile plant materials were recovered from meristem culture of *Sequoiadendrum gigantum* (Lindl.) J.Buchh. (Monteuuis 1991). In guava, position of the bud explant influenced shoot proliferation efficiency with production of intermediate buds and higher number of shoots (Shekafandeh and Khoshkhui 2008) while in passion fruit, plants having intermediate buds showed higher plant height during proliferation without difference in leaf number per shoot (Faria et al. 2007) but intermediate buds of *Corylus avellana* L. were superior with higher viability and increased shoot proliferation (Hand et al. 2016). In *Aloe vera* (L.) Burm.f. and *Punica granatum* L. tissue cultures, repeated culturing of the micro shoot improved frequency of shoot morphogenesis and elongation up to the 4th subculture cycle (Kanwar et al. 2015; Soni and Kanwar 2016) while decrease was observed in the case of *Rubus pubescens* L. after 3rd subculture cycle (Debnath 2004). For Cherry (Gisela 6 and Fereley Jaspi), the morphogenesis index was stable from 2nd cycle to the 4th (Vujovic et al. 2012).

Leaves morphogenesis

Succesful growth of micro shoot in the *in vitro* condition during shoot proliferation is determined by the degree of expanded leaves development and their photosynthetic efficiency (Tsukaya 2002). In the present experiment, because the shoot cultures were serially subcultured, the number of leaves per micro shoot were noted during the subcultures. It was observed that the influence of cytokinins on shoot morphogenesis and elongation also reflected on the leaves differentiation (Fig. 2d). Cytokinins in the culture medium may cause decrease in leaf limb and Chl production accompanied with increased gas exchange in culture vessels, reducing ethylene accumulation essential for leaves development (Abeles et al. 1992; Silva et al. 2017).

Rhizogenesis

In juvenile trees, vigorous *in vitro* rooting is often encountered but, adventitious rooting ability becomes reduced at the maturity (Pijut et al. 2011). However, adventitious rooting potential may be improved after rejuvenation of adult trees, and efficiency of auxins supplemented in the culture medium for root morphogenesis is determined by the interaction between micro shoot and endogenous hormones concentration levels (Zhang et al. 2017). Some changes also related to vegetative maturation, and on the basis of plant morphology, becomes reversed through rejuvenation to restore rooting ability of micro shoot, with physiological reversion of aging regarded pre-requisite to the successful achievement (Hackett 1985). In the present study, pale-yellowish root formation with long root hairs were observed within 2–3 wks culture of micro shoots on solid half basal MS medium added with the auxins at variable degree, which was on the basis of auxin supplemented in the culture medium (Fig. 1o–p). However, IBA and NAA were more efficient on root differentiation over IAA at the lower levels, while higher produced unhealthy roots; medium supplemented with the auxins below 1.0 mg L^{-1} produced characteristic slender roots while higher produced thick and branched roots (Fig. 1o–p). It is possible that the supplemented IBA promoted adventitious rooting by the number of roots formed per micro shoot without improving root growth but, was difficult to assess as the shoots were maintained through single subculture in rooting medium. In *Pinus massoniana*, efficiency of rooting ability was remarkably improved by successive grafting and rejuvenation after several subcultures of micro shoots with increased rooting efficiency in 20 subculture cycles but, declined after 30 subcultures with IBA been the most efficient auxin among others tested (Wang and Yao 2019).

Pigment molecules and cellular osmolytes content analysis from the leaves of micro shoots

In higher plants, leaf colouration is attributed to its various pigments of Chl, Cars and anthocyanins that plays the role of harvest and transfer of light energy in the antenna systems, the charge separation and electron transport function in reaction centers (Fromme et al. 2003). Hence, Chl and Cars accumulation in plants correlates with the physiological growth stage due to their influence on photosynthetic activity to meet the metabolic demand of active cells (Samanta et al. 2017). The Chl is a green plant pigment molecule that contains magnesium ion at the porphyrin ring center and occurs in two forms; Chla is a photosynthetic pigment that absorbs blue, red and violet wavelength in the visible spectrum while Chlb complete the absorption spectrum of the Chla. Because *in vitro* cultivated plants have limited

photosynthetic ability, mainly influenced by the culture condition and PGRs that influence their growth and development, they are characterised by high phenotypic plasticity in response to the growth condition, with leaves playing prominent role in light energy capture for survival under the condition (Isah 2015; Isah and Umar 2018; Isah 2019b). Variation in leaf coloration under the condition is determined by Chl contents of species, the maturity stage of leaves and other physiological growth conditions with ratio of Chla and b serving as indicators of developmental stage of photosynthetic apparatus of leaves. In the present study, the two morphometric leaves types observed in *R. serpentina* micro shoot cultures (DG and PG) were subjected to pigment molecule analysis aimed at identifying their possible differences in photosynthetic efficiency that influence many biochemical pathways, with overall effect on growth and development under the *in vitro* condition, through the analysis of photosynthetic parameters of Chla and b and Cars, and showed variation in the parameters studied (Fig. 3a). Chlorophyll and Cars content are to some extent dependent on mineral elements presence in growth medium of plants, in addition to other environmental physiological factors, with PGRs playing substantial role (Sardoei 2014). In the present study, higher Chlb content of the PG leaves over Chla provides explanation to the differential coloration observed, as it is associated with yellow-green coloration in leaves of plants (Devlin and Witham 1997). Another possible reason for the differential Chl accumulation in the two (2) leaves can be explained by inactive or less light harvesting complexes in the PG leaves that may play role in the lower adaptation of the shoots to *in vitro* condition under the ambient light intensities of the culture vessel, that in the overall, led to the formation of small and slender leaf in PG leaves shoot cultures over the DG ones (Fig. 1n–o and h–m). Reduction in Chla and b contents observed in PG leaves of this species could also be attributed to degradation of Chls into their derivative pheophytins a and b due to mg^{2+} elimination (Boekel 1999).

Carotenoids are accessory photosynthetics pigment molecules that cover visible spectrum light absorption not utilized by Chls, while xanthophylls and lutein have higher bioaccessibility when carotenes concentration is low (Zaripheh and Erdman 2002), and forms major Cars that accumulate in young leaves under shade or low light conditions of the *in vitro* culture (Yoon et al. 2012). They can be synthesized during unfavourable growth condition for the scavenging of free radicals, and as light-harvesting pigment quenchers of Chl state, and singlet O_2 species among other functions (Safafar et al. 2015). During first 14 days of aclimatization of *in vitro* regenerated *R. serpentina* plantlets, Alatar (2015) reported reduction in Cars production but showed increase at 28 days, suggesting increase in their accumulation in response to changing environment of the plants. It is important to note that the spectrophotometric method used in the present experiment enables only estimation of total Cars in the leaves samples without description of the individual carotenoids such as β-carotene, lutein, zeaxanthin and lycopene that are possibly present in the extracts (Jodlowska and Latala 2011). This also suggest that relative ratio of Chl and Car could indicate stress level of the leaves and their photosynthetic function (Filimon et al. 2016) with higher ratio serving protective and beneficial function to growth under the *in vitro* condition of plants (Niroula et al. 2019).

Leaf coloration can also be used to determine the stress levels of plants under the *in vitro* condition with accumulation of osmolyte molecules of TSS, glycinebetaine and proline serving as marker compounds (Fig. 3b). The organic osmolytes are accumulated in response to osmotic stress imposed by the *in vitro* culture condition to plants (Rameeh et al. 2017; Annunziata et al. 2019). Under such condition, proline could serve as carbon, nitrogen storage sink and as scavenger of free radicals (Chinnusamy et al. 2005) or stabilizer of subcellular structures and buffer to cellular redox potential (Chinnusamy et al. 2005; Kavi Kishor et al. 2005). For glycinebetaine whose synthesis in cells is dependent on high ATP consumption and showed higher accumulation in PG leaves (Fig. 3b), they are osmoprotectants that stabilize protein quaternary structures and high ordered membrane state for alleviating osmotic stress to plant tissues under the *in vitro* condition (Lokhande et al. 2010; Ali et al. 2020). These results suggests that the osmolyte molecules of TSS, glycinebetaine and proline were accumulated at higher levels in the PG leaves over the dark ones through upregulation in the expression of genes involved in their biosynthesis (Sakamoto and Murata 2000) to enable the plants adjust to the condition of *in vitro* culture growth (Norris 1968; Isah 2015) at differential levels and to variably cope with the condition, as reflected by the two leaf morpho-types observed in the cultures. In the present study, high correlation in the accumulation of Chls and Cars also suggests that increase in the pigment molecules reflects the stable *in vitro* growth stage of the micro shoots, corresponding to irradiance and plant growth developmental physiology of the culture vessel. Besides positive correlation on the Chla and b content observed in the leaves, moderate and positive correlation on the Chla and Cars levels were recorded, with also weak net relative content of proline and TSS in the leaves. Dark-green leaves were characterised with higher Chl contents and thickened texture over the PG ones (Fig. 11n–o and h–m), possibly due to increased thickness of spongy mesophyll influenced by the lighting source that translates into higher production of photosynthetic pigments in the leaves.

Results of the present study suggests that nodal segment explant type and collection season influenced *in vitro* rejuvenation and micropropagation efficiency in *R. serpentina* plants. The strength of the culture medium and amended PGRs also influenced rejuvenation events, resulting in formation of shoots bearing two morphometric leaves types, possibly influenced by the elemental composition strength of the cultivation medium. Analysis of pigment molecules and cellular osmolytes showed their differential *in vitro* culture adaptive role in plant physiological state, based on differential accumulation in the tissues analyzed.

Abbreviations

MS
Murashige and Skoog medium
PGRs
plant growth regulators
BAP
N^6-benzylaminopurine

TDZ
thidiazuron
Kin
kinetin
NAA
naphthalene acetic acid
IAA
indole 3-acetic acid
IBA
indole 3-butyric acid
DG
dark-green leaves
PG
pale-green leaves
Chl
Chlorophyll
TSS
total soluble sugars
FW
fresh weight
PSM
plant secondary metabolites
ANOVA
analysis of variance

Declarations

Conflict of interest statement

Author declares that conflict of interest does not exist in the manuscript contents.

Ethics approval and consent to participate

The research work was carriedout in compliance with ethical standard that did not involve the use of humans.

Consent for publication

Authors declares having consent to publish the manuscript contents.

Availability of data and material

Available on reasonable request to corresponding author.

Competing interests

None exist

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Authors' contributions

TI and NZ conceived the research idea, performed experiments, wrote, edited and approved the manuscript contents.

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Figures

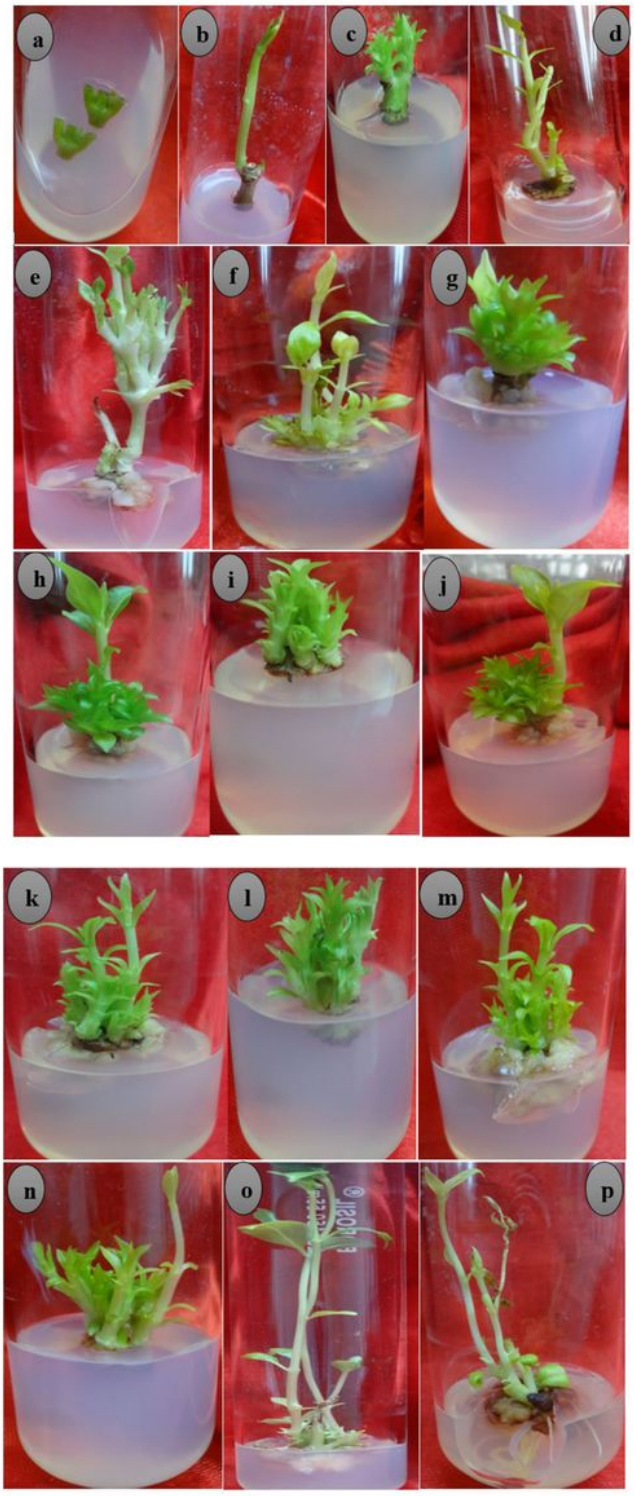

Figure 1

stages of *in vitro* rejuvenation of nodal segment explants in *Rauvolfia serpentina* L.; **a** terminal bud-derived nodal segment explant, **b–c** basal offshoot-derived nodal segment explant, **d–e** micro shoots produced on solid TDZ-amended medium, **f–g** BAP-induced micro shoot, **h–i** Kin-induced micro shoots on medium added with 1.0, 2.0 and 1.0 mg L^{-1} respectively, **h–n** successive subculture of the micro shoots at different stages on solid medium supplemented with cytokinins, **o–p** rhizogenesis on half basal solid MS medium added with NAA and IBA at 1.0 and 0.5 mg L^{-1} respectively. *bars:* **a**–d 1.5 mm, **e**–**p** 2.0 mm.

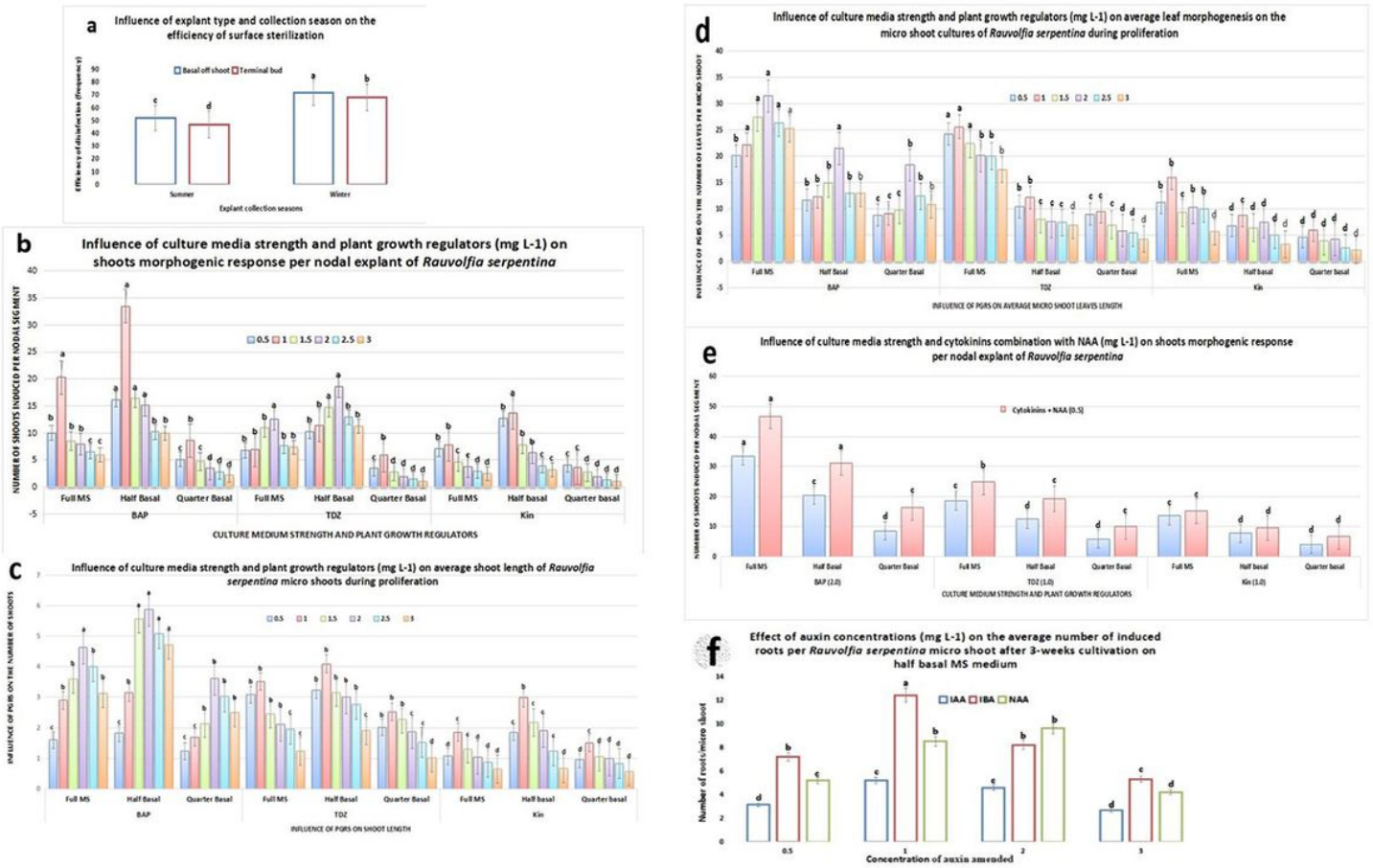

Figure 2

Data on explant rejuvenation micropropagation experiments.

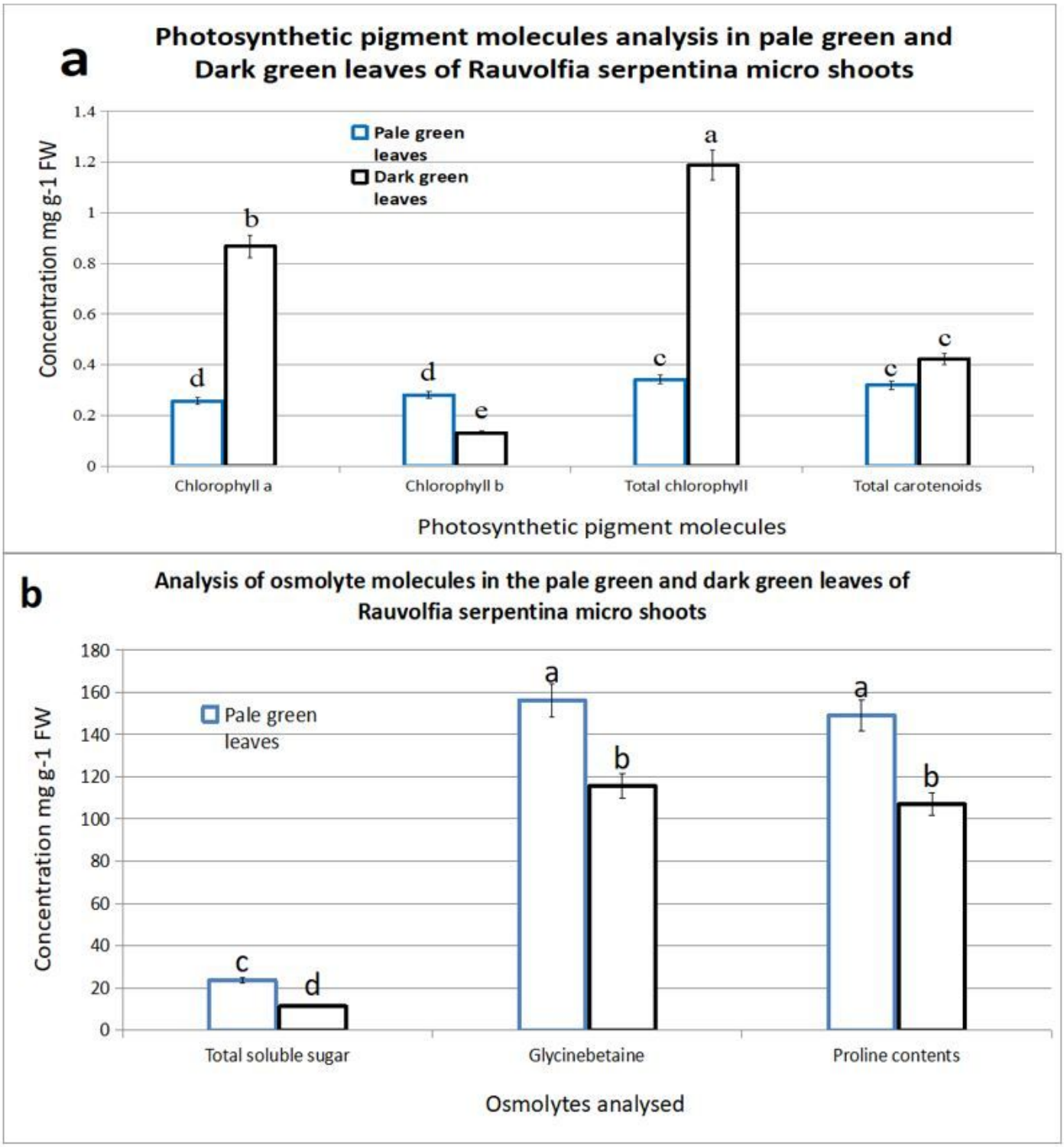

Figure 3

Data on pigment and osmolytes molecules analysis experiments.