Synergistic of growth regulators and salicylic acid enhances shoot proliferation and acclimatization in Sugar beet (*Beta vulgaris* L.)

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Abstract

The establishment of a fast method to in vitro multiplication and adaptation of sugar beet (*Beta vulgaris* L., would allow us to multiply the crop rapidly and breed it. Several treatments of α-naphthaleneacetic acid (NAA; 0.5, 1, 1.5 mg l^{-1}), 6-benzyladenine (BA; 0.5, 1, 2 mg l^{-1}) and salicylic acid SA; (5, 10,15 mg l^{-1}) were subjected to the explants based on percent callus growth shoot length root growth and survival plantlets derived from hardened hypocotyl epicotyl and petiole segments taken from seedlings in vitro The longest shoots were obtained from hypocotyl, epicotyl, and petiole explants cultured on medium supplemented with NAA (1.5 mg L^{-1}) + BA (2.0 mg L^{-1}) + SA (15 mg L^{-1}), with mean shoot lengths of 4.8, 4.7, and 4.5 cm, respectively. Furthermore, root length was significantly increased in response to the treatment containing NAA (1.5 mg L^{-1}) + BA (1.0 mg L^{-1}) + SA (5 mg L^{-1}), indicating a pronounced stimulatory effect of this growth regulator combination on root development. respectively, when compared to the control. The treatment NAA1.5 + BA2 mg L^{-1} SA showed the maximum amount of both callus proliferation and regrowth of shoots (200 - 350% and 16.8-32.4% respectively). The highest number of microshoots formed (2.5–2.7 shoots per explant, +167–180%) was recorded in NAA1 + BA2 + SA10 mg L^{-1}. The best acclimatization was reported for the treatment NAA 1.5 + BA2 + SA 15 mg L^{-1} with an acclimatization rate of 68–75% compared to the control, representing an increase of 24–68%. The results in the present study confirm that NAA-BA-SA combinations can aid in improving regeneration, rooting, and acclimatization, while providing an effective pathway to further develop methods for the large-scale propagation of sugar beet for biotechnological purposes.

Keywords: Sugar beet, In vitro regeneration, Shoot proliferation, Acclimatisation.

Introduction

Sugar beet (*Beta vulgaris* L.) is a crop of key agricultural and industrial importance as a major source of sucrose in temperate areas and as a significant crop in the global sugar industry [46]. In addition to its significance as a major sucrose-producing crop, sugar beet (*Beta vulgaris* L.) has substantial economic and agro-industrial importance due to the utilization of its biomass and processing by-products as livestock feed and substrates for bioenergy production. Consequently, the crop contributes to diversified agricultural production systems and supports multiple value-added economic applications. However, sugar beet will continue to be troubled by challenges stemming from traditional breeding and production due to stresses from biotic and abiotic factors (both conditions limiting crop growth) and by the limited genetic diversity available for future adaptation, as well as by the challenge to breed some genotypes that are

grown successfully in commercial production and cultivated without the use of traditional breeding and production methods [9,51,38]. These ongoing challenges require the development of new approaches to crop improvement, and among the various strategies, in vitro culture and plant tissue regeneration have become essential.

Morphogenesis and in vitro regeneration allow for the quick propagation of elite genotypes, facilitate genetic transformation, and contribute to the production of disease-free plant material [40]. These processes rely on both the external application of plant growth regulators (PGRs) and the inherent balance of PGRs, which lead to cellular differentiation and organogenesis [54]. Among the plant growth regulators, Naphthaleneacetic acid (NAA), which is an auxin (a synthetic product), promotes cell division and elongation, as well as root development [12]. 6-Benzyladenine (BA) is a synthetic type of plant growth regulator (PGR) which is a cytokinin that mainly promotes cell division, proliferation and shoot development. Depending on the relative concentration of the two and on the particular morphogenic response of the plant tissue, its relation with auxins is synergistic or antagonistic. [34]. Salicylic acid (SA) is a signaling molecule that has antifungal capabilities as part of plant defense mechanisms, but as new evidenced has shown, it may promote morphogenesis and enhanced stress tolerance in vitro [45].

The intricate relationship between auxins, cytokinins, and the phytohormone salicylic acid (SA), is critical to the regulation of morphogenic pathways [21]. Hormonal interactions determine what cultured cells may develop into, and thus, hormonal interactions decide whether callus, organogenesis, or somatic embryogenesis takes place in a micropropagation study [39]. In addition, recent investigations showed the bilateral application of naphthaleneacetic acid (NAA) and benzyladenine (BA) can substantially enhance regeneration efficacy across various sugar beet cultivars by increasing the auxin-to-cytokinin ratio [15,2,49]. Combined with SA, which has been demonstrated to improve tissue viability and mitigate oxidative stress, improves regeneration capacity through survival of the tissue [11] Despite these reported improvements, the underlying physiological and biochemical mechanisms governing the interactive effects of naphthaleneacetic acid (NAA), 6-benzyladenine (BA), and salicylic acid (SA) remain insufficiently elucidated, particularly in sugar beet (*Beta vulgaris* L.). , incomplete knowledge of mechanisms and recalcitrance to regeneration and genotype-dependent responses to manipulation remain a considerable barrier.

Past studies have addressed either the role of auxins in tissue culture, or the role of cytokinins in tissue culture with some emerging literature suggesting a potential effect of SA on morphogenic outcomes [21,6], but there are not many comprehensive studies on a combination of these hormones, particularly in vitro conditions optimized for sugar beet. Without this research, regeneration protocols are difficult to determine and wider applications of biotechnology are limited in the sugar beet improvement context.

Accordingly, this research wants to highlight the interactive influence of NAA,BA, and SA on morphogenesis in vitro and regeneration ability in sugar beet (*Beta vulgaris* L.) This research aims to promote our understanding of hormonal regulation in morphogenesis pathways, gain information within the field of plant developmental biology, and promote the use of tissue culture work with sugar beet and relatives.

Material and Methods

Plant material and sterilization

Plant material of *Beta vulgaris* L. (sugar beet) was collected from cultivated fields in the fields Department of Horticulture and Landscape Engineering, (31°52′ N, 56°1′ E), College of Agriculture and Forestry, University of Mosul.The seeds were rinsed thoroughly in running tap water for 30 min and then agitated with a few drops of mild detergent, surface sterilized in 70% (v/v) ethanol for 30s then transferred to the solution of l/100 volume of bleach containing 2.5% (w/v) sodium hypochlorite and supplemented with a drop of Tween-20 per 100 ml added as wetting agent, for agitation between 10 min -15 min. The seeds were subsequently rinsed 3 to 5 times in sterile distilled water, following sodium hypochlorite treatment (to eliminate any disinfectant residues). Sterilized seeds were aseptically germinated on hormonefree Murashige and Skoog (MS) medium in a growth chamber at 25 ± 2 °C for 16 h with light intensity of 40350mol m^{-2} s^{-1}. Hypocotyls, epicotyls and petiole segments (1-2 cm in length) were isolated from the seedlings on sterile medium at 7 to10 days after germination.

The inoculated explants were initially pre-incubated to facilitate regeneration and subsequently cultured on a semi-solid Murashige and Skoog (MS) basal medium supplemented with different concentrations of plant growth regulators, including naphthaleneacetic acid (NAA) at 0.5, 1.0, and 1.5 mg L^{-}

6-benzyladenine (BA; 0.5, 1, and 2 mg l^{-1}) and salicylic acid (SA;5, 10 and 15 mg l^{-1}). All media were autoclaved at 121°C for 15 min, and they all had an agar (0.8%, w/v) added to the liquid medium. Cultures were incubated at 25 ± 2 °C with a 16 hour photoperiod at 40-50 µmol m^{-2} s^{-1} illuminate with fluorescent lights. The sterilization procedure and initiation cultures were conducted in a laminar flow cabinet to maintain aseptic conditions.

Callus Formation in Application PGRPs

Beta vulgaris L. seedlings were germinated on hormone-free MS medium in an aseptic manner and explants, as hypocotyls, epicotyls, and petiole segments (1–2 cm in length) were excised from 7-10 day-old seedlings. To preserve the integrity of meristematic tissues, explants were cut into two or more equal pieces of approximately equal size. Explant were cultured on solid MS medium 0.8% agar; with 30 g l^{-1} sucrose supplemented with plant growth regulators at various concentration of NAA (0.5, 1 and 1.5 mg l^{-1}) combined to BA (1, 1.5 and 2 mg l^{-1}) as well as SA(BA) (5, 10 and 15mg mg l^{-1}), when needed. The cultures were kept in the growth chamber at 25 °C ± 1 °C, under a photoperiod of 16 h (cool-white light [700-800 lux]). All cultures were sub-cultured monthly. After three months, data was recorded for callus fresh weight, callus induction rate and embryo callus development.

Salicylic acid Elicitor Treatment

SA was tested at three concentrations (5, 10 and 15 mg l^{-1}) for its effect on biomass accumulation and metabolite production. SA was first dissolved in methanol, then dissolved in deionized water, sterilized by filtration into the medium For the control treatments, an equivalent volume of filter-sterilized methanol was incorporated into the culture medium. Ten-day-old cell suspensions were filtered, and two grams of fresh biomass were transferred into 50 mL flasks containing 30 mL of MS medium supplemented with BAP and NAA. Cultures were incubated in the dark on a rotary shaker at 120 rpm at 23–25 °C. After three days, the SA and control solutions were added, and the cultures were treated for an additional four days [36].

Growth Measurement

The shoot length (cm) was determined as the average length of shoots per explant, and measured with a digital caliper. The length (cm) of the root was also measured from the bottom of the shoot to end tip of longest root. Number of roots per shoot were recorded, in addition to these measurements. **Renegade shoots were then carefully removed from culture**

vessels and the number of leaves and the number of branches per shoot was manually counted under a stereomicroscope. Only fully expanded leaves and clearly distinguishable branches were included in the count. The results were expressed as the **mean number of leaves and branches per explant** for each treatment. All measurements were carried out under aseptic conditions, with each treatment consisting of at least ten explants and three biological replicates.

Fresh Callus Weight

To measure the fresh callus weight (g), callus tissue is first carefully removed from the culture medium using sterile forceps. Excess culture media that is firmly attached to the callus is dabbed off carefully using sterile filter paper in an effort to not include the weight of the extra liquid. The cleaned callus is then promptly weighed on a precision analytical balance and recorded in grams (g), as this measurement represents the fresh weight of the callus as water-saturated mass, or an un-dried weight, and can be used for comparing growth on different culture conditions.

Shoot Regeneration Percentage

Shoot regeneration percentage was determined by culturing explants, such as leaf discs or petiole segments, on an appropriate shoot-induction medium under controlled *in vitro* conditions. Following the emergence of shoots, the proportion of explants exhibiting successful shoot regeneration was recorded and expressed as a percentage of the total number of cultured explants. that produced at least one shoot in relation to acceptable time (4weeks) of culture, by taking the number of explants that produced shoots and dividing that by the total number of explants cultured and multiplying that by 100. Also note the average number of shoots produced per explant to see the proliferation [24].

Root Induction Percentage

For root induction, regenerated shoots (3–4 cm long) were separated and transferred to culture medium. Cultures were maintained at 25 ± 2°C and under a 16.8 h photoperiod (light/dark). After 21 days of culture, the percentage of shoots that developed roots was recorded for each treatment. The percentage of root induction was calculated using the following formula:

$$\text{Root induction \%} = \frac{\text{Number of shoots with roots}}{\text{Total number of cultured shoots}} \times 100$$

Each treatment included at least 10 shoots, and the experiment was conducted in three replicates. All observations were made under aseptic conditions.

Callus Induction Percentage

The percent callus induction was calculated by the number of explants that had developed a visible callus over total number of explants inoculated and multiplied by 100. Cotyledonary Explants (hypocotyls, epicotyledon, petioles) were cultured on MS-based media for the different treatments described and after a certain culture time (usually three/four weeks) number of explants with callus induction was recorded [24]. The value was expressed as:

$$\text{Callus induction \%} = \frac{\text{Number of explants forming callus}}{\text{Total number of explants inoculated}} \times 100$$

Mean Number of Microshoots

The regenerated microshoots developed from each explant were counted with in four weeks of culture. The average microshoot number per explant was determined by the total number of regenerated shoots divided by the total count of explants cultured in reliability and potentiation treatments:

$$\text{Mean Number of Microshoots} = \frac{\text{Total number of shoots regenerated}}{\text{Total number of explants cultured}} \times 100$$

All cultures were maintained under a 16/8 h (light/dark) photoperiod at 25 ± 2°C. Each treatment consisted of at least 10 explants and three replicates.

Acclimatisation Percentage

After rooting, the in vitro regenerated plantlets were taken out from the culture

containers aseptically and washed with sterile distilled water to get rid of agar. Plantlets were transplanted into plastic pots with sterilized peat moss, perlite and vermiculite (1:1:1) mixture and kept under high humidity condition (covered with clear polyethylene bag) for one week to ten days after transferring. The attenuation of humidity was stepwise, with covers partially opened during 2 weeks in this study. Light (1000–1500 lux with 16/8 h light/dark photoperiod) and temperature conditions (25 + 2 °C) were uniform across all assays.

$$\text{Acclimatisation \%} = \frac{\text{Number of surviving plantlets}}{\text{Total number of transferred plantlets}} \times 100$$

After 4 weeks, the number of surviving plantlets was recorded, and the acclimatisation percentage was calculated using the following formula:

Statistical analysis

Statistical analysis was performed using the SAS software (version 9.4), in which a comparison of means of the different treatments was investigated using the least significant difference (LSD) test at the α=0.05 significance level. Hierarchical clustering and Pearson correlation analysis were accomplished in R (version 4.2.3, using appropriate packages for R).PCA was generated with XLSTAT package.

Results and Discussion

Optimizing Shoot and Root Development Across Explants

The results indicated that the treatment comprising 1.5 mg L^{-1} NAA + 2.0 mg L^{-1} BA + 15 mg L^{-1} SA produced the highest mean shoot length across all explant types. The mean shoot lengths reached 4.8 cm in hypocotyledon-derived explants and 4.7 cm in epicotyledon-derived explants.), and 4.5 cm (petiole explant), as shown in Table 1. Rooting response was highest among explants treated with 1.5 mg l^{-1} NAA + 1.0 mg l^{-1} BA + 5 mg l^{-1} SA. In this treatment, the root lengths for hypocotyledon, epicotyledon, and petiole explants reached 6.4, 7.2, and 6.3 cm, respectively, while the control explants had root lengths of 1.5, 2.1, and 1.8 cm, which reflects an increase in root length of 326.6%, 242.8%, and 250%, respectively (Table 1). The rooting response for leaf and petiole explants was influenced by explant type and hormone treatment. For the control, root lengths were 0 cm for leaf explants and 3 cm for petiole explants. Treatments with NAA 1 mg l^{-1} + BA + SA enhanced rooting, but the greatest root lengths were NAA1+BA2+SA10, with 5 cm for leaves and 6 cm for petioles, which were increases of 500% for leaves and 100% for petioles compared to control, respectively. Limiting the concentration of NAA (0.5 mg l^{-1}) or not having an appropriate balance of BA/SA resulted in no rooting or limited rooting. The addition of NAA at 1.5 mg l^{-1} improved rooting but did so only in combination with the BA/SA combination (Table 1). The differences in shoot and root responses in explants likely arise from the differential responses of tissues to auxin–cytokinin ratios. In particular, higher levels of NAA may facilitate root initiation [31], BA stimulates shoot extension while SA serves as a modulator of stress signaling and organogenesis, which can all promote optimal growth depending on the type of explants and the hormonal balance [53]. [43] presented a successful protocol to regenerate plants from nodal cultures of *Aspidosperma polyneuron* seedlings using BA and NAA producing overall high shoot regeneration rates and rooting percentages, especially from hypocotyl explants. Similarly, [10] established a system for breaking seed dormancy and regenerating plants in perennial vulneraria milkvetch, with significant shoot regeneration observed in hypocotyl and epicotyl explants treated with Kin-NAA and BAP-NAA. [1] showed that node explants of *Lagerstroemia speciosa* responded much better to 0.25 µM NAA and 1.0 µM BA than to the other treatments. They

produced the highest number of shoots and adequate rooting. Similarly, the rooting of explant was significantly improved with the NAA and BA treatments on MS media as found from another study that reported the highest root production with 0.4 mg l^{-1} NAA and 1 mg l^{-1} BA [7]. Furthermore, the addition of NAA and BA has been proven to produce adventitious shoots in *Oncidium tigrinum*. The most effective treatments for adventitious shoot production were determined to be the MS medium supplemented with 1 to 2 mg l^{-1} BA and 0.1 mg l^{-1} NAA [35]. A study using twigs from *Tetraclinis articulata* as explants, determined optimal multiplication rates were achieved on medium supplemented with 4.0 mg l^{-1} BA and 0.05 mg l^{-1} NAA [22].

Synergistic Effects of NAA, BA, and SA on Explant Regeneration

The treatment that produced the highest amount of plant tissue induction in every type of explant was NAA1.5 + BA2 + SA15, which resulted in hypocotyledon 1.5 g, epicotyledon 1.7 g, and petiole 1.8 g, or a 200%, 183%, and 350% increases respectively, when compared with control samples (Fig. 1a). The enhanced callus induction response may be attributed to the synergistic interaction between the auxin NAA and the cytokinin BA, which together stimulate cellular proliferation and promote callus formation. [4], and presence of SA may promote dedifferentiation and proliferation through alteration of processes regulating growth and stress-responsiveness. The hormone combinations containing BA alone and 2,4-D, 4-CP, and BA yielded comparable results towards the efficiency of callus induction and proliferation, with the highest percentage of callus induction occurring at the correct ratio with hormones for each of the exact plant species studied and documented [44]. Additionally, the highest frequencies of callus induction also occurred given plant tissue on a media with the concentration of 2,4-D in N6 media. The same concentration of hormones initiated the optimum shoot initiation at the appropriate concentrations of BA and NAA [14]. Similarly, other studies found that the addition of NAA with BA at different concentrations also produced significant results in callus induction, with the specific combination that had the most significant induction of callus percentage [47]. Nonetheless, some studies also showed that the combination of BA and NAA was less effective in inducing callus than some of the combinations [23]. Additionally, the type of explant and growth conditions (light vs dark) also had an effect on how effective these hormonal combinations were at inducing callus [47].

Moderate regeneration was achieved with low NAA (0.5 mg l^{-1}) and moderate BA (0.5 mg l^{-1}) reaching 8.3% in hypocotyledon and 7.8% in petiole. Higher BA rates (1-2 mg l^{-1}) and low NAA inhibited shoots completely. The maximum regeneration was achieved at NAA1.5+BA2+SA15 mg l^{-1}, with 16.8%, 25.6% and 32.4% hypocotyledon, epicotyledon, and petiole respectively (Fig. 1a). The regeneration of the shoot varies based on the combination of both BA and NAA, along with their concentrations. Higher NAA concentrations will induce callus formation within the explants; however, this will take longer for the shoot to regenerate, and ultimately fewer shoots produced per explant [26]. With respect to shoot regeneration in Sedum sarmentosum, MS medium containing 8.0 µM BA produced greater shoot regeneration frequency and more shoots per explant [29]. The interactive effect of different growth regulators, including BA, NAA, and IBA, is important for shoot regeneration and root induction. A medium that consisted of 2 mg l^{-1} IBA and 0.1 mg l^{-1} NAA was determined to be the best hormonal balance for root induction [17] These findings demonstrate that successful plant tissue culture is determined not only by the type of plant growth regulator employed but also by its concentration and the specific

combination of growth regulators used. Optimizing these factors is therefore essential for achieving efficient and reproducible *in vitro* plant regeneration. The explant type can also act as signaling molecules during the regeneration process; for example, leaf and internode explants showed a significant difference in callus induction and shoot regeneration further supporting the idea that the explant type is an additional variable to consider when optimizing regeneration protocols [25].

Efficient Callus and Root Formation via NAA-BA-SA Treatments

The highest root induction percentage was recorded in explants cultured under the treatment combination of 1.5 mg L^{-1} NAA + 0.5 mg L^{-1} BA + 15 mg L^{-1} SA. Root formation reached 74% in hypocotyledon explants, 85% in epicotyledon explants, and 74% in petiole explants. These values represented a significant increase in root induction compared with the control treatment, which exhibited only 18–25% root formation.

(Fig. 2a). The combination of NAA and 6-BA can be used to induce adventitious bud differentiation. However, the optimal NAA concentration for inducing root formation is highly dependent on the specific requirements of the explant and the prevailing *in vitro* culture conditions. [52]. SA is known to play an important effect in regulating different plant growth and stress response processes. Studies have suggested that SA may influence root development and architecture depending on a given concentration [41]. Moreover, SA increased formation of root aerenchyma, which is advantageous for plants adaptive responses to waterlogging [30]. SA has been described to interact with other plant hormones and signaling pathways. For example, it has been shown to influence the synthesis and transport of auxin, which are important for root development [41]. A protocol was developed for *Neolamarkia Cadamba* using cotyledonary petioles and hypocotyls, where TDZ combined with NAA was found to be the most effective cytokinin for bud induction [19]. Likewise for Scaevola sericea, combinations of BA and NAA were successful for shoot organogenesis as well as somatic embryogenesis using leaf explants and root explants [33]. Researchers have also shown that combinations of cytokinins and auxins have significant effects on both the rates of shoot and root induction, as well as on the quality of the shoots and roots. For instance, Bailey and NAA were achieved successful shoot organogenesis and proliferation in Corydalis saxicola, using leaf and petiole explants [33]. In addition, a combination of NAA and BA was found to be the best for direct bud organogenesis of Begonia rex for buds [18].

In this study, callus induction was highest for explants treated with NAA1+BA1+SA10, with hypocotyledon explants reaching 62% callus induction, followed by epicotyledon (59%) and petiole (53%) explants, compared to 10–23% in the control (Fig. 2b). This is likely due to the combination of auxins and cytokinins, such as NAA and BA, which is integral to callus induction as it stimulates cell division and enlargement [42], Although NAA alone is capable of inducing callus formation, its application in combination with BA can enhance the efficiency and accelerate the rate of callus induction. This finding suggests that an appropriate balance between auxins and cytokinins is critical for optimizing callogenesis and achieving effective *in vitro* tissue culture responses. [16]. [28] made the observation that the internode of the Cardinal variety of potato takes different times for callus initiation at different concentrations of NAA, with a longer initiation time at higher concentration. A similar finding was presented during other trials where NAA was used in combination with BAP for callus and shoot formation [5,32]. Another factor that contributes to callus induction efficiency is the explant type. For example, explants taken from internodes induced 100% callus induction

with identified environmental applications [5,28], while leaf explants also proved to have great potential for callus induction [28,20]. In addition, it has been demonstrated that including NAA with other growth regulators (BAP, KN) can enhance both callus induction, and the quality of the callus, as well as shoot multiplication and rootlet production [5]. In addition to phenotypic effects, concentrations of NAA have also been shown to be effective depending on concentration, and the limited studies referenced in this publication suggest concentration can have serious effects. For instance, a lower concentration of NAA has been less effective, in some cases [3], whereas optimal concentrations were found to have a greater percentage of callus induction [5,28].

Enhanced Microshoots Growth and Acclimatisation

In hypocotyledon explants, those that were treated with NAA1+BA2+SA10 yielded the highest number of microshoots of 2.5, as well as the highest number of microshoots in epicotyledon and petiole explants with 2.6 and 2.7 microshoots respectively.. To consider the differences statistically, there was a significant increase in the number of microshoots to 2.5 (167% increase in hypocotyl), 2.6 (173% increase in epicotyl) and 2.7 (180% increase in petiole) compared to control explants with 1.1, 0 and 1.5 microshoots respectively (Figs. 3a). This indicates that NAA1+BA2+SA10 is the best treatment for shoot proliferation. This notable increase in the number of microshoots is due to the combined effect of auxin (NAA) and cytokinin (BA) [50], to work together to regulate cell division and differentiation, and SJ likely supported organogensis through regulating stress-related signaling related to stimulating shoot proliferation [2]. In a similar way, BA in combination with NAA improved the regeneration capacity of M. officinalis, and the highest shoot formation was obtained with axillary bud explants [48]. Also, unlike in a previous example described, the use of both SA and BA in organ culture for Acinos graveolens caused substantial changes in growth parameters and essential oil components, though all but one treatment decreased growth parameters, as compared to the control [37] In a separate study, different concentrations of salicylic acid (SA) and chitosan were reported to exert concentration-dependent effects on the accumulation of therapeutic secondary metabolites in shoot cultures of *Swertia paniculata*. These findings provide further evidence that exogenously applied elicitors and growth regulators can modulate secondary metabolite biosynthesis and consequently influence the production of pharmacologically valuable compounds. [27].

The NAA1.5+BA2+SA15 treatments had the greatest respiration (75% acclimatisation in hypocotyledon, 68% in epicotyledon, and 72% in petiole), indicating an increase in acclimatisation of 39%, 68%, and 24%, respectively, compared to control (54%, 0%, 58%) for hypocotyledon, epicotyledon, and petiole (Fig. 3b). NAA1.5 + BA2 + SA15 appears to be the most effective combination treatment to stimulate acclimatisation in each explant type. The better acclimatisation rates are likely a result of both higher auxin and cytokinin levels promoting strong root and shoot growth [31], and because SA encourages stress tolerance, enabling greater chances of survival during the transition from in vitro conditions to ex vitro [13,2]. Recent research pertaining to acclimatization of plant material and in vitro studies has identified pertinent outcomes using certain growth regulators. For example, the use of N6-benzylaminopurine (BA) at 0.1-1.0 mg l^{-1} concentration in Murashige and Skoog media was effective in initiating cultures from node explants in Senna artemisioides, a native legume in Australia [8]. In addition, [8] observed optimal proliferation in cultures (2.4 shoots per explant) at a concentration of BA of 0.5 mg l^{-1}. This

suggests that BA is a contributing factor in the multiplication phase when working with in vitro plant culture[8].

Heatmap and PCA Analysis of Hormonal Effects

The heatmap illustrates that the hormone combination NAA0.5+BA0.5+SA10 mg l^{-1} had a significant positive correlation with acclimatisation across all explants (Hypocotyledon, Petiole, and Epicotyledon) suggested the consideration for enhanced likelihood of success in the acclimatisation stage in ex vitro conditions. NAA1+BA0.5+SA10 mg l^{-1} showed a significant positive correlation with acclimatisation and root induction within Epicotyledon indicating improved probability of survivability and rooting in this tissue type. These results indicate that explant responses to hormones can be tissue dependent and that specific combinations can encourage both root induction and simultaneously help acclimatise explants (Fig. 4a).

The PCA analysis showed that all treatments except the control treatment and the specified hormone combinations, NAA0.5+BA0.5+SA5 mg l^{-1}, NAA0.5+BA2+SA10 mg l^{-1}, NAA0.5+BA1+SA15 mg l^{-1}, NAA0.5+SA1+SA5 mg l^{-1}, NAA0.5+BA0.5+SA10 mg l^{-1}, NAA1.5+BA0.5+SA5 mg l^{-1}, NAA1+BA2+SA15 mg l^{-1}, and NAA0.5+BA2+SA15 mg l^{-1}, indicated a positive correlation with the measured parameters of acclimatisation, shoot means, the number of microshoots, callus induction, leaf/branch variation, root length, rhizogenesis, shoot recovery, and overall fresh callus weight. This suggests that the effective hormone combinations work together to improve both shoot and root growth, callus development, and the performance of the explant overall, illustrating both tissue and concentration dependent hormone responsiveness during explant development (Fig. 4b,c,d).

Table 1. Effect of different concentrations of NAA in combination with BA and salicylic acid on in vitro from three explant types (hypocotyledon, epicotyledon, and petiole) of *Beta vulgaris* L.

leaf/branch			Root length (cm)			Shoot means (cm)			Treatment (mg l^{-1})
Petiole	Epicotyledon	Hypocotyledon	Petiole	Epicotyledon	Hypocotyledon	Petiole	Epicotyledon	Hypocotyledon	
3^{cd}	0^{f}	2^{d}	1.8^{q}	2.1^{pq}	1.5^{qr}	2.8^{f}	0.0^{k}	3.2^{e}	Control
3^{cd}	2^{d}	3^{cd}	2.5^{o}	2.1^{pq}	1.3^{r}	2.6^{g}	2.4^{gh}	2.1^{i}	$NAA_{0.5}+BA_{0.5}+SA_{5}$
2^{d}	3^{cd}	4^{c}	1.8^{q}	1.8^{q}	2.1^{pq}	2.2^{h}	1.8^{j}	2.1^{i}	$NAA_{0.5}+BA_{0.5}+SA_{10}$
3^{cd}	4^{c}	3^{cd}	2.3^{p}	2.2^{p}	2.1^{pq}	2.4^{gh}	2.6^{g}	2.4^{gh}	$NAA_{0.5}+BA_{0.5}+SA_{15}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{1}+SA_{5}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{1}+SA_{10}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{1}+SA_{15}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{2}+SA_{5}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{2}+SA_{10}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{0.5}+BA_{2}+SA_{15}$
2^{d}	4^{c}	3^{cd}	2.8^{n}	2.6^{no}	2.7^{no}	2.4^{gh}	2.3^{h}	2.2^{h}	$NAA_{1}+BA_{0.5}+SA_{5}$
1^{e}	2^{d}	3^{cd}	3.6^{l}	3.5^{l}	3.1^{m}	3.4^{d}	2.6^{g}	2.5^{gh}	$NAA_{1}+BA_{0.5}+SA_{10}$
2^{d}	3^{cd}	2^{d}	4.2^{k}	4.5^{ij}	4.1^{k}	3.4^{d}	3.5^{d}	3.2^{e}	$NAA_{1}+BA_{0.5}+SA_{15}$
5^{b}	4^{c}	3^{cd}	5.8^{f}	5.2^{hi}	4.5^{ij}	3.5^{d}	4.2^{b}	4.1^{b}	$NAA_{1}+BA_{1}+SA_{5}$
5^{b}	5^{b}	6^{ab}	4.4^{j}	4.8^{i}	4.7^{i}	2.8^{f}	4.1^{b}	3.5^{d}	$NAA_{1}+BA_{1}+SA_{10}$
4^{c}	5^{b}	6^{ab}	4.6^{ij}	4.1^{k}	5.2^{hi}	2.4^{gh}	2.6^{g}	2.5^{gh}	$NAA_{1}+BA_{1}+SA_{15}$
6^{ab}	5^{b}	6^{ab}	4.1^{k}	4.7^{i}	5.2^{hi}	3.8^{c}	3.7^{c}	2.5^{gh}	$NAA_{1}+BA_{2}+SA_{5}$
6^{ab}	5^{b}	7^{a}	3.5^{l}	6.5^{c}	3.5^{l}	3.5^{d}	3.6^{d}	3.4^{d}	$NAA_{1}+BA_{2}+SA_{10}$
0^{f}	0^{f}	0^{f}	0.0^{s}	0.0^{s}	0.0^{s}	0.0^{k}	0.0^{k}	0.0^{k}	$NAA_{1}+BA_{2}+SA_{15}$

5^{b}	3^{cd}	0^{f}	6.5^{c}	6.5^{c}	0.0^{s}	2.3^{h}	2.1^{i}	0.0^{k}	$NAA_{1.5}+BA_{0.5}+SA_{5}$
4^{c}	2^{d}	6^{ab}	3.6^{l}	7.4^{a}	6.3^{d}	2.3^{h}	2.5^{gh}	2.4^{gh}	$NAA_{1.5}+BA_{0.5}+SA_{10}$
2^{d}	3^{cd}	2^{d}	6.5^{c}	6.8^{b}	5.4^{h}	2.5^{gh}	2.2^{h}	2.1^{i}	$NAA_{1.5}+BA_{0.5}+SA_{15}$
2^{d}	1^{e}	4^{c}	6.3^{d}	7.2^{ab}	6.4^{cd}	2.3^{h}	2.4^{gh}	2.6^{g}	$NAA_{1.5}+BA_{1}+SA_{5}$
1^{e}	2^{d}	3^{cd}	5.8^{f}	5.9^{f}	6.8^{b}	2.3^{h}	2.2^{h}	2.5^{gh}	$NAA_{1.5}+BA_{1}+SA_{10}$
1^{e}	3^{cd}	2^{d}	6.5^{c}	6.3^{d}	6.1^{e}	2.4^{gh}	2.3^{h}	2.1^{i}	$NAA_{1.5}+BA_{1}+SA_{15}$
5^{b}	6^{ab}	5^{b}	5.6^{g}	5.8^{f}	6.5^{c}	4.5^{ab}	4.2^{b}	3.5^{d}	$NAA_{1.5}+BA_{2}+SA_{5}$
7^{a}	5^{b}	6^{ab}	6.1^{e}	6.3^{d}	4.8^{i}	3.2^{e}	3.3^{e}	3.5^{d}	$NAA_{1.5}+BA_{2}+SA_{10}$
5^{b}	6^{ab}	4^{c}	5.8^{f}	5.6^{g}	5.4^{h}	3.6^{d}	4.7^{a}	4.8^{a}	$NAA_{1.5}+BA_{2}+SA_{15}$

Means followed by different letters are significantly different at $p \leq 0.05$ according to the LSD test.

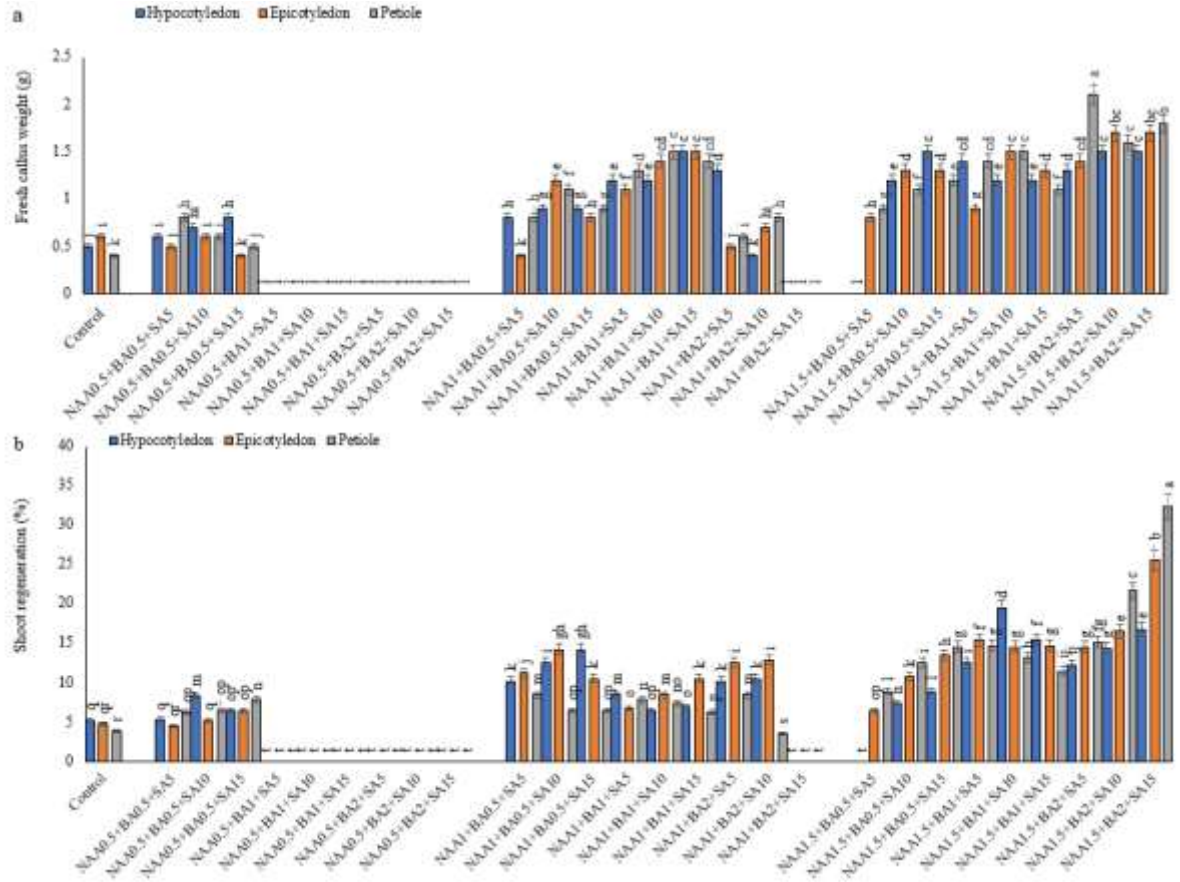


Fig 1. Effect of different concentrations of NAA (0.5, 1.0, and 1.5 mg l^{-1}) in combination with BA (1.0, 1.5, and 2.0 mg l^{-1}) and salicylic acid (SA; 5, 10, and 15 mg l^{-1}) on in vitro shoot regeneration from three explant types (hypocotyledon, epicotyledon, and petiole) of *Beta vulgaris* L.: **(a)** fresh callus weight and; **(b)** shoot regeneration. Data represent mean ± SE (n = 3). Means followed by different letters are significantly different at $p \leq 0.05$ according to the LSD test.

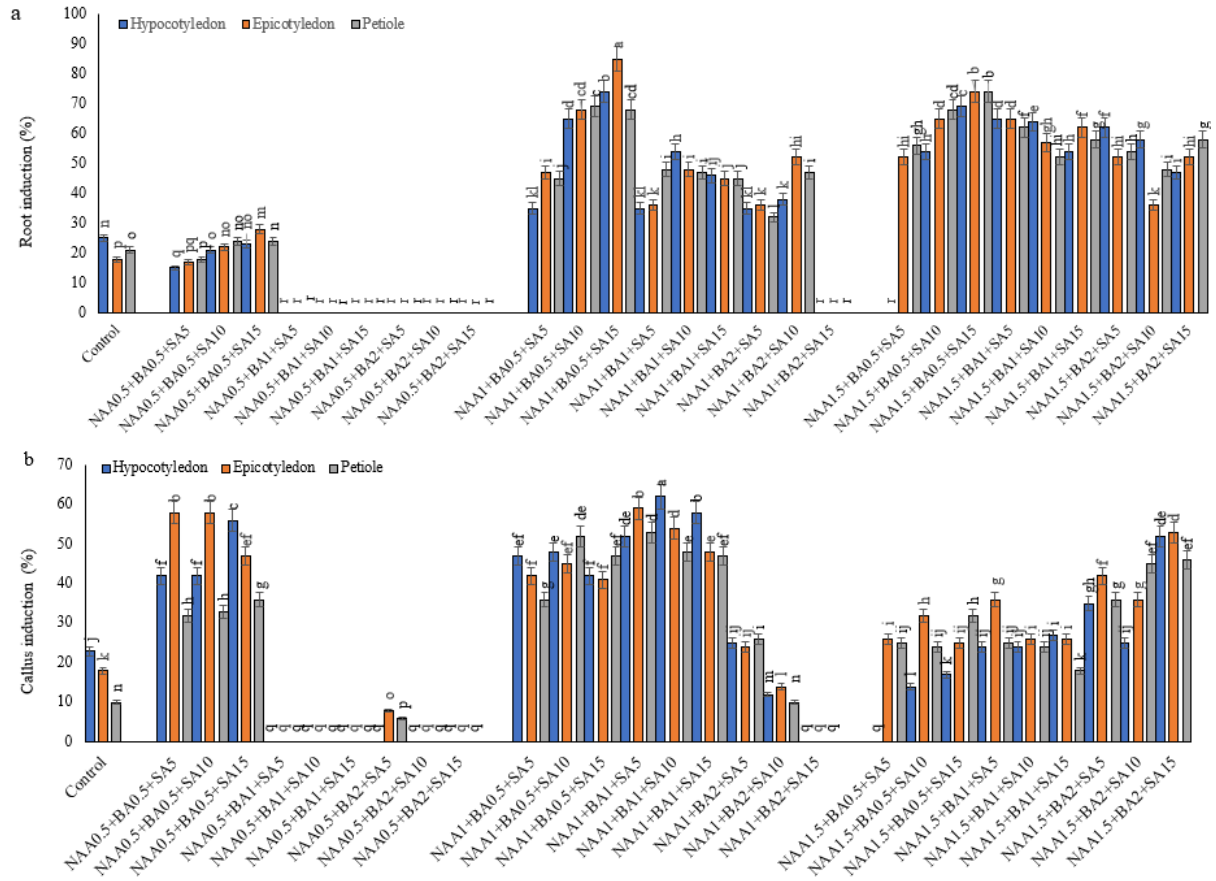


Fig 2. Effect of different concentrations of NAA (0.5, 1.0, and 1.5 mg l^{-1}) in combination with BA (1.0, 1.5, and 2.0 mg l^{-1}) and salicylic acid (SA; 5, 10, and 15 mg l^{-1}) on in vitro shoot regeneration from three explant types (hypocotyledon, epicotyledon, and petiole) of *Beta vulgaris* L.: **(a)** root induction and; **(b)** callus induction. Data represent mean ± SE (n = 3). Means followed by different letters are significantly different at $p \leq 0.05$ according to the LSD test.

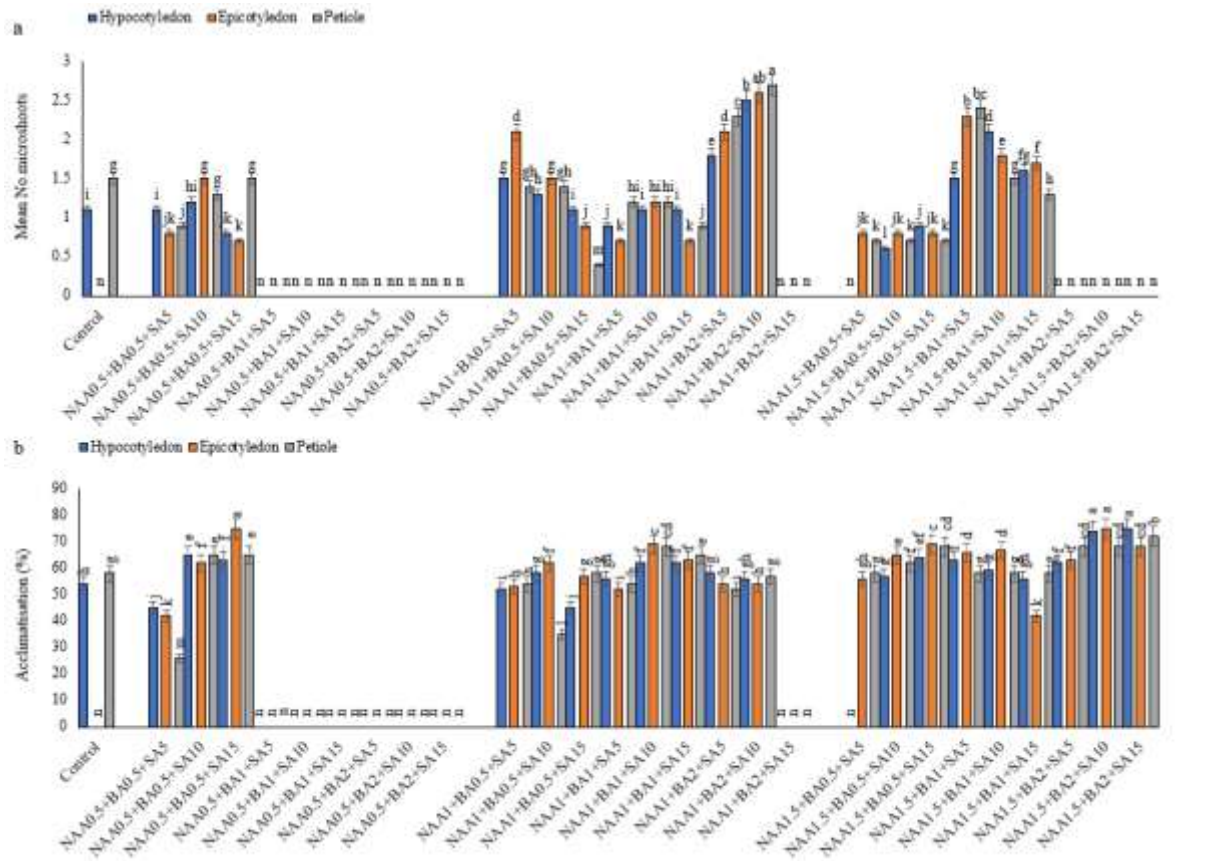
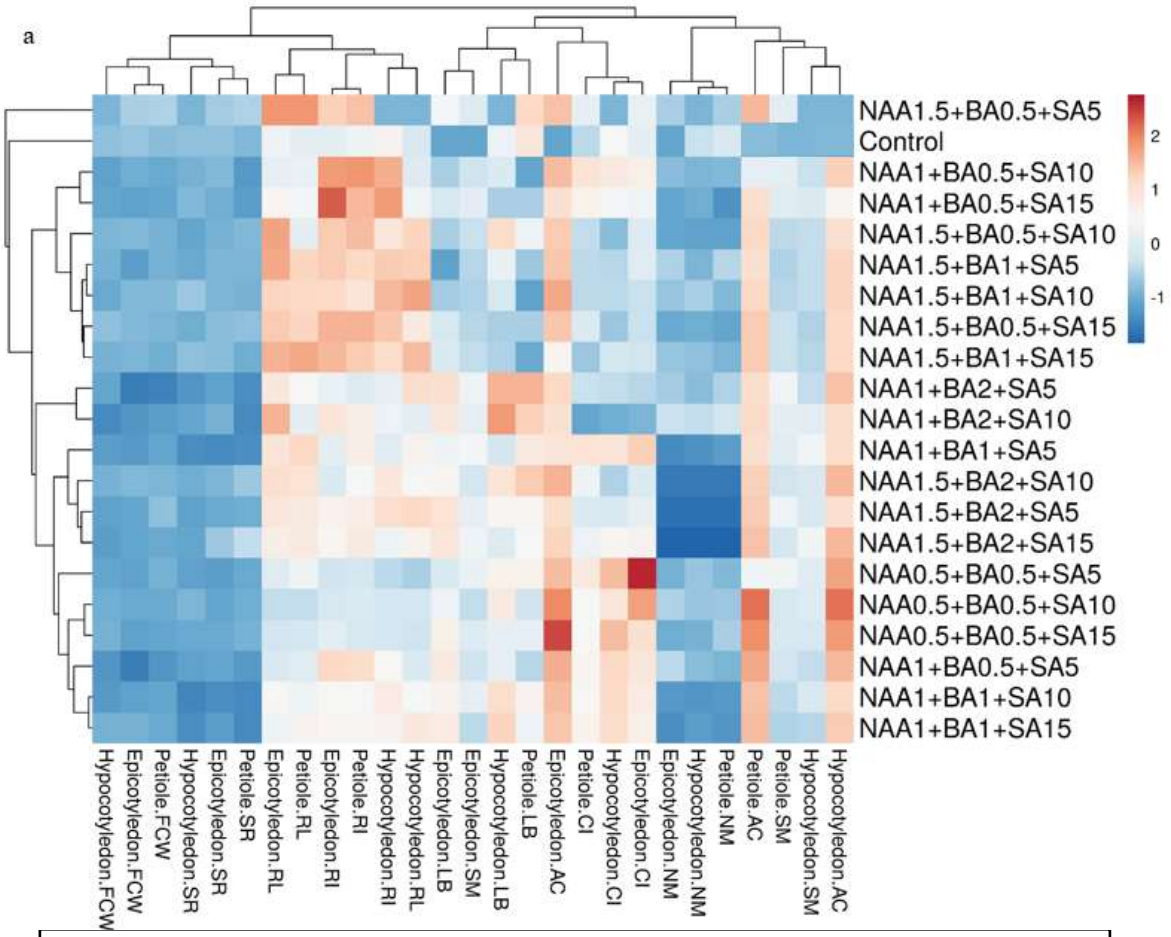
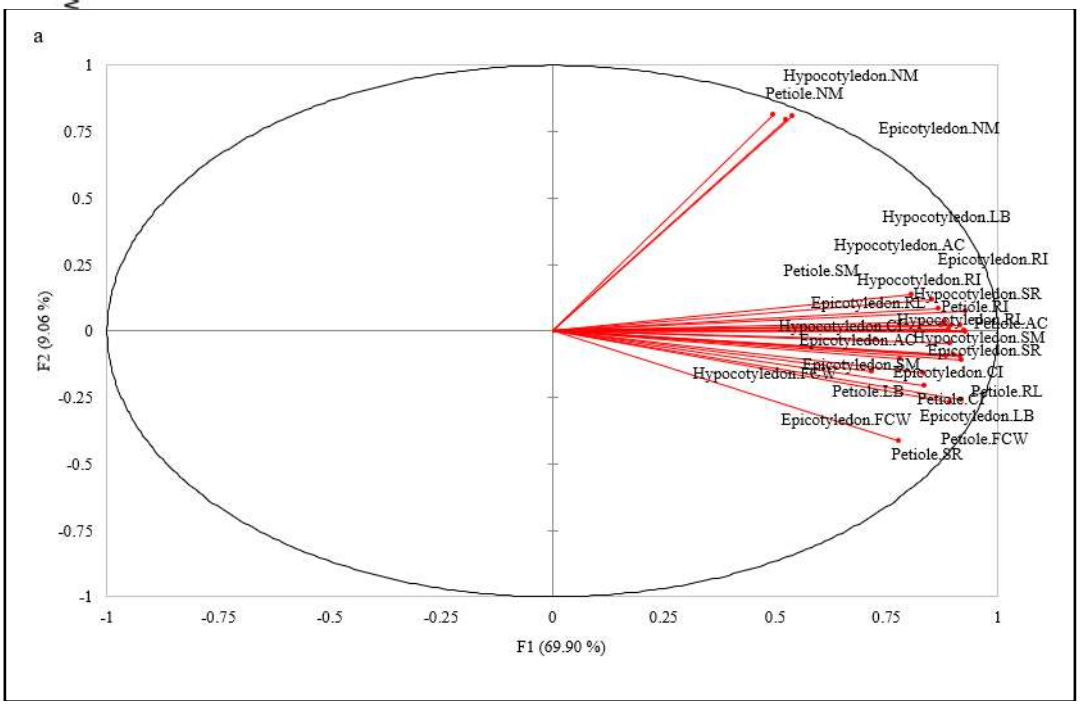


Fig 3. Effect of different concentrations of NAA (0.5, 1.0, and 1.5 mg l^{-1}) in combination with BA (1.0, 1.5, and 2.0 mg l^{-1}) and salicylic acid (SA; 5, 10, and 15 mg l^{-1}) on in vitro shoot regeneration from three explant types (hypocotyledon, epicotyledon, and petiole) of *Beta vulgaris* L.: **(a)** mean no microshoots and; **(b)** acclimatisation. Data represent mean ± SE (n = 3). Means followed by different letters are significantly different at $p \leq 0.05$ according to the LSD test.


a
NAA1.5+BA0.5+SA5
Control
NAA1+BA0.5+SA10
NAA1+BA0.5+SA15
NAA1.5+BA0.5+SA10
NAA1.5+BA1+SA5
NAA1.5+BA1+SA10
NAA1.5+BA0.5+SA15
NAA1.5+BA1+SA15
NAA1+BA2+SA5
NAA1+BA2+SA10
NAA1+BA1+SA5
NAA1.5+BA2+SA10
NAA1.5+BA2+SA5
NAA1.5+BA2+SA15
NAA0.5+BA0.5+SA5
NAA0.5+BA0.5+SA10
NAA0.5+BA0.5+SA15
NAA1+BA0.5+SA5
NAA1+BA1+SA10
NAA1+BA1+SA15
2
1
0
-1
Hypocotyledon.FCW
Epicotyledon.FCW
Petiole.FCW
Hypocotyledon.SR
Epicotyledon.SR
Petiole.SR
Epicotyledon.RL
Petiole.RL
Epicotyledon.RI
Petiole.RI
Hypocotyledon.RI
Hypocotyledon.RL
Epicotyledon.LB
Epicotyledon.SM
Hypocotyledon.LB
Petiole.LB
Epicotyledon.AC
Petiole.CI
Hypocotyledon.CI
Epicotyledon.CI
Epicotyledon.NM
Hypocotyledon.NM
Petiole.NM
Petiole.AC
Petiole.SM
Hypocotyledon.SM
Hypocotyledon.AC


a
F2 (9.06 %)
F1 (69.90 %)
1
0.75
0.5
0.25
0
-0.25
-0.5
-0.75
-1
Hypocotyledon.NM
Petiole.NM
Epicotyledon.NM
Hypocotyledon.LB
Hypocotyledon.AC
Epicotyledon.RI
Petiole.SM
Hypocotyledon.RI
Hypocotyledon.SR
Epicotyledon.RL
Petiole.RI
Hypocotyledon.RL
Petiole.AC
Hypocotyledon.CI
Epicotyledon.AC
Hypocotyledon.SM
Epicotyledon.SR
Epicotyledon.SM
Epicotyledon.CI
Hypocotyledon.FCW
Petiole.LB
Petiole.RL
Petiole.CI
Epicotyledon.FCW
Epicotyledon.LB
Petiole.FCW
Petiole.SR

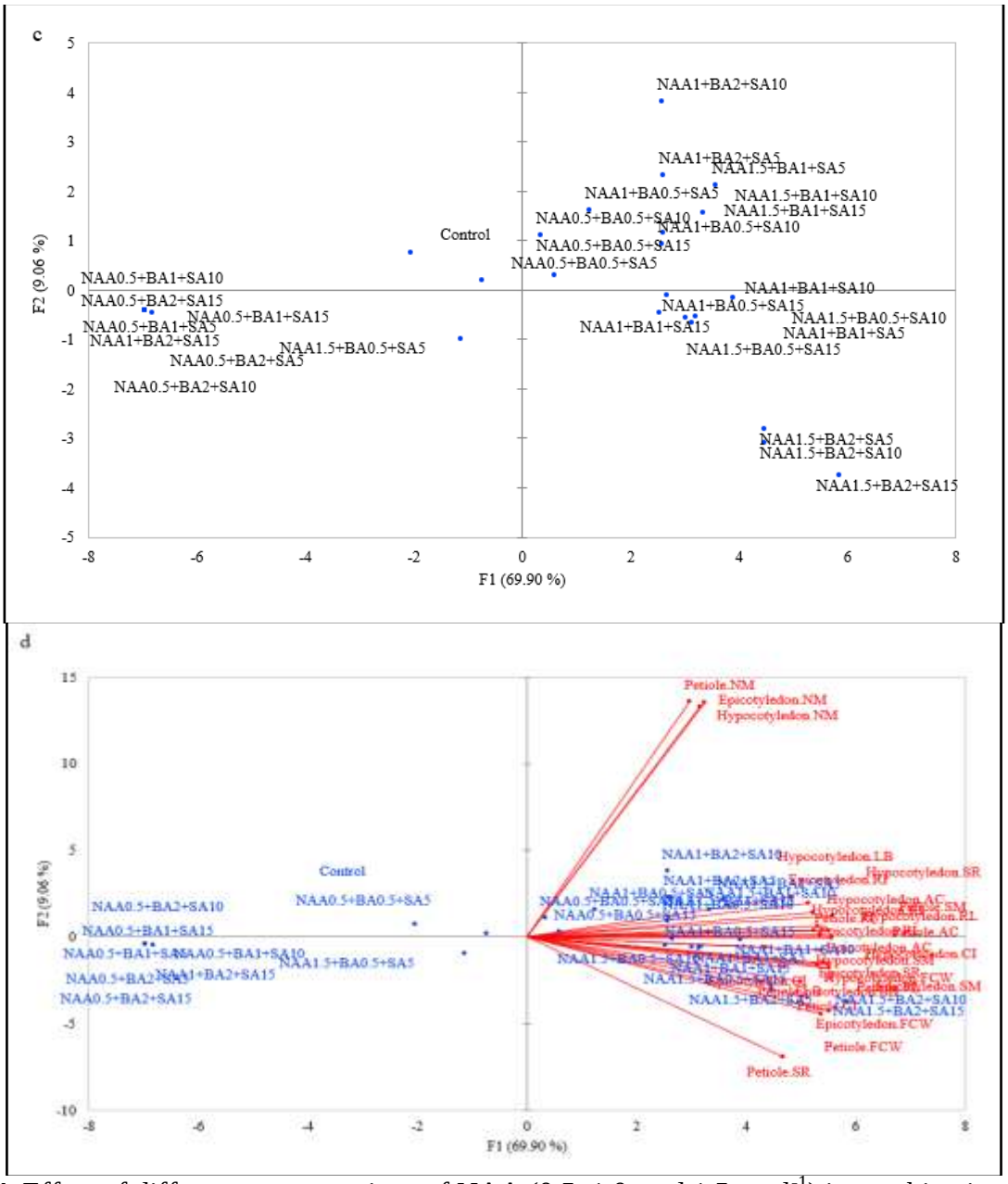


Fig 4. Effect of different concentrations of NAA (0.5, 1.0, and 1.5 mg l^{-1}) in combination with BA (1.0, 1.5, and 2.0 mg l^{-1}) and salicylic acid (SA; 5, 10, and 15 mg l^{-1}) on in vitro shoot regeneration from three explant types (hypocotyledon, epicotyledon, and petiole) of *Beta vulgaris* L. **(a)** Hierarchical clustering analysis (HCA) of Pearson's correlation coefficient (r) values of variable traits, where the color scale that indicates r coefficient values (r = 1.5 to -1) indicates positive (red) and negative (blue) correlations and; **(b,c,d)** Principal component analysis (PCA). Parameters include: AC: acclimatisation; SM: Shoot means; NM: no microshoots; CI: callus induction; LB: leaf/branch; RL: root length; RI: root induction; SR: shoot regeneration; FCW: fresh callus weight.

Conclusion

The study's findings indicated that a range of concentrations of NAA, BA and SA led to significant outcomes for in vitro regeneration, rooting and acclimatization of sugar beet (*Beta vulgaris* L.) explants. The combination treatment of 1.5 mg l^{-1} NAA + 2 mg l^{-1} BA + 15 mg l^{-1} SA produced the greatest shoot lengths and

callus production, and the highest acclimatization rates (68-75%). Meanwhile, rooting was positively regulated by 1.5 mg l^{-1} NAA + 1 mg l^{-1} BA + 5 mg l^{-1} SA, while NAA 1 mg l^{-1} +BA 2 mg l^{-1} + SA 10 mg l^{-1} produced the highest number of microshoots. Overall, the experiments establish an efficient and effective protocol for mass propagation of this species, and establish the potential for utilization and regeneration of plantlets to inform genetic improvement and biotechnological applications. Research moving forward can then establish long-term growth performance, stress tolerance and field establishment of regenerated plantlets as part of subsequent Knowledge Transfer steps to improve confinement quality/quantity of sugar beet production on horticultural scales.

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