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Red light and biostimulants containing arbuscular mycorrhizal fungi enhance ajmalicine and serpentine production in *Catharanthus roseus* under indoor conditions

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Indoor farming offers optimal conditions for high and stable ajmalicine and serpentine yields in *Catharanthus roseus*, enabling precise control over environmental *stimuli* such as light and biostimulants. This study aimed to evaluate, for the first time, the combined effects of LED light spectrum and a biostimulant containing arbuscular mycorrhizal fungi on ajmalicine and serpentine production in indoor-grown *C. roseus*. Following a 60 days pre-treatment under white LEDs, plants received eight treatments: white (W, control), red (R), blue (B), red-blue (RB, R:B = 6:1) LED light, and their combinations with the biostimulant (BS). Samples were collected before treatments (T0) and at harvest (T1, 92 days after pre-treatment). R and RB significantly increased aerial fresh weight compared to W and B, with no significant differences in plant height relative to W. Although no mycorrhizal colonization occurred, BS significantly increased plant height, shoot fresh weight, total dry weight, leaf serpentine concentration, and total serpentine yield (+ 34.4%) compared to the untreated condition. When combined, R and BS had a synergistic effect on ajmalicine, resulting in its highest concentration and yield. Under this treatment, ajmalicine concentration and yield were significantly higher than in all other treatments (+ 144.3% and + 138.0% compared to the W control, respectively), except for the yields under B and RB with BS, which were comparable to those under R with BS. From T0 to T1, total serpentine yield increased by 2277.1%, while ajmalicine yield rose by 716.0%. In conclusion, the combined application of R and BS, followed by harvest after a 92 days treatment period, is the most effective strategy to optimize ajmalicine and serpentine production while ensuring vigorous yet size-controlled plant growth.

Keywords Controlled environment agriculture, LED light, Biostimulants, Arbuscular mycorrhizal fungi (AMF), *Catharanthus roseus*, Secondary metabolites

Catharanthus roseus (L.) G. Don, commonly known as Madagascar periwinkle, is a tropical perennial plant, of the *Apocynaceae* family[1], renowned for producing terpenoid indole alkaloids (TIAs) with significant pharmaceutical applications[2]. Among these, vinblastine (VBL) is the most well-known due to its widespread use as an anticancer drug[3]. In addition to VBL, *C. roseus* produces other medically important TIAs, such as ajmalicine (AJM) and serpentine (SRP), which are clinically employed in the treatment of hypertension and other circulatory disorders[4]. Recently, AJM has been reported to possess neuroprotective activity, positioning it as a potential candidate for Alzheimer's treatment[5]. AJM is found exclusively in the roots, while SRP is present in both the roots and aerial parts of the plant[6]. The market value of AJM is significant, estimated at approximately

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$8.9 million per kilogram[7]. SRP is valued as a source of AJM since it can be converted into AJM using a reducing agent such as NaBH4[8].

In most Mediterranean regions, where *C. roseus* is cultivated as an ornamental or medicinal crop, the plant cannot survive winter temperatures due to its tropical origin[9]. This limitation makes year-round outdoor cultivation challenging[10]. Moreover, its specialized metabolism is strongly influenced by cultivation methods, environmental conditions, and genetic variability[11]. As a result, stable production of TIAs in open-field, including AJM and SRP, is difficult. Controlled indoor farming systems offer a promising solution, as they enable precise management of environmental *stimuli*, including light and biostimulants. Such control can enhance AJM and SRP accumulation while ensuring stable supply over time [11].

Precise light regulation in indoor farming has been revolutionized by light-emitting diode (LED) technology. LEDs offer the possibility to optimize light conditions and improve plant growth and performance[12]. In particular, by modulating light spectrum according to photoreceptor characteristics, LEDs can regulate light signal transduction pathways, promoting the biosynthesis of specialized metabolites[13–15]. Research on *C. roseus* in indoor farming systems has primarily focused on optimizing lighting conditions, both in terms of quality (spectrum) and quantity (intensity), to enhance the production of VBL and its precursors, vindoline (VDL) and catharanthine (CAT)[10,16–20].

While the influence of light on VBL, VDL, and CAT biosynthesis is relatively well-documented, less is known about its impact on AJM and SRP, particularly in relation to light spectrum. Light has been shown to promote the accumulation of AJM and SRP[21]. However, most studies investigating light effects on AJM and SRP have been conducted in vitro and have primarily focused on binary light conditions (i.e., light vs. darkness), photoperiod duration, or intensity, often yielding contradictory results[22]. Some studies reported increased accumulation of AJM and SRP under light exposure [21], while others suggested higher concentrations in dark conditions[23]. Only a few have specifically examined light spectral composition, and these were limited to in *vitro* systems[24,25]. To date, no comprehensive *in planta* study has addressed the effects of specific LED light spectra on the production (concentrations and yields) of AJM and SRP.

Beyond lighting, biostimulants, especially those containing arbuscular mycorrhizal fungi (AMF), have also shown considerable promise in enhancing biomass production and specialized metabolite accumulation in medicinal plants, including Madagascar periwinkle[26,27]. AMF are known to improve nutrient uptake (particularly phosphorus), increase stress tolerance, and stimulate the production of specialized metabolites[28]. Studies on *C. roseus* have shown that AMF inoculation increases mass yield [29] and promotes the synthesis of VBL in the leaves as well as SRP and AJM in the roots.[30] In addition to AMF, commercial biostimulant formulations frequently consist of complex mixtures that include other beneficial microorganisms (e.g., endosymbionts like *Rhizobium* and plant-growth-promoting rhizobacteria), along with bioactive substances such as humic compounds, organic nitrogen compounds, seaweed extracts, potassium salts, phosphates, and phytohormones. These formulations have been widely used to promote plant growth and stimulate the production of specialized metabolites in various plant species, including *C. roseus* [31].

Although the individual effects of LED lighting (particularly spectral composition) and AMF-containing formulations, on alkaloid production in *C. roseus* have been studied to some extent, research in this area is still at an early stage. In particular, their combined influence remains largely unexplored. Even less is known about how specific LED spectra and AMF-containing biostimulants interact to regulate the *in planta* accumulation of AJM and SRP. Hence, this study investigates the synergistic effects of LED spectra and AMF-containing biostimulants on AJM and SRP production in *C. roseus*, with the aim of identifying novel strategies to maximize the yields of these valuable alkaloids.

Results

AFM colonization

A complete lack of mycorrhizal colonization was observed in all root samples analyzed, both at T0 and T1.

Analysis of variance (ANOVA) results

ANOVA results are summarized in Table 1. Only significant results are analyzed and discussed. Specifically, in cases where at least one of the main factors was significant and the interaction involving them was also significant (as observed for AJM concentration and AJM yield), only the interaction (i.e., all light spectrum:BS combinations) was analyzed and discussed to avoid a potentially misleading interpretation of the results. Conversely, when the interaction was not significant but one or both main effects were significant, the main effects were analyzed and discussed.

Effect of light spectrum and BS on plant growth

Leaf, shoot and aerial FW

Figure 1A, B and D show the main effect of light spectrum for leaf, shoot and aerial FW, respectively. RB light produced the highest leaf and aerial FW (25.9 ± 0.7 g plant^{-1} and 35.5 ± 0.8 g plant^{-1}, respectively), while R light resulted in the highest shoot FW (11.04 ± 0.3 g plant^{-1}). Conversely, the lowest values for all three variables were observed under W light (13.0 ± 0.7 g plant^{-1} for leaf FW, 6.8 ± 0.3 g plant^{-1} for shoot FW and 19.8 ± 0.8 g plant^{-1} for total aerial FW). In particular, for leaves, RB light resulted in a significantly higher FW than all the other light treatments (+99.2% compared to W); R light also produced a significantly higher leaf FW compared to W and B light (+71.5% relative to W). Regarding shoots, R light significantly increased FW compared to all the other light treatments (+62.1% compared to W light). For the aerial part of the plant, both RB and R light treatments were statistically comparable, but both resulted in a significantly higher FW compared to the other light treatments (+79.3% and +68.2% compared to W, respectively).

	Light spectrum	BS treatment	Light spectrum:BS treatment
Leaf FW (g plant^{-1})	***	ns	ns
Shoot FW (g plant^{-1})	***	*	ns
Aerial FW (g plant^{-1})	***	ns	ns
Leaf DW (g plant^{-1})	ns	**	ns
Root DW (g plant^{-1})	ns	*	ns
Total DW (g plant^{-1})	ns	***	ns
Plant height (cm)	***	**	ns
Leaf SRP concentration (µg g^{-1} DW)	ns	*	ns
Leaf SRP yield (µg plant^{-1})	ns	**	ns
Root SRP concentration (µg g^{-1} DW)	ns	ns	ns
Root SRP yield (µg plant^{-1})	ns	*	ns
Mean SRP concentration (µg g^{-1} DW)	ns	ns	ns
Total SRP yield (µg plant^{-1})	ns	*	ns
AJM concentration (µg g^{-1} DW)	***	**	***
AJM yield (µg plant^{-1})	ns	***	**

Table 1. Results from the two-way ANOVA. Effect of light spectrum and AMF-containing biostimulant (BS) treatment on the growth, and SRP and AJM production in *C. roseus*. Significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***), ns = not significant differences.

The main effect of the BS treatment (Fig. 1C) revealed that it significantly influenced only the shoot FW. Specifically, the BS treatment resulted in a significant increase in shoot FW compared to the untreated condition (+7.0%).

Leaf, root and total DW

The main effect of the BS treatment revealed that leaf (Fig. 2A), root (Fig. 2B), and total (Fig. 2C) DW all increased significantly under BS treatment compared to the untreated condition, with respective increases of 25.6%, 25.5%, and 25.6%.

Plant height

The main effect of light spectrum (Fig. 3A) revealed that the greatest plant height was achieved under B light (27 ± 0.4 cm), while the lowest height was observed under RB light (21.8 ± 0.4 cm). Specifically, B light resulted in a significantly greater plant height compared to all other light treatments, (+17.9% compared to W light). Additionally, R light produced a significantly greater plant height compared to RB light (+11.0%) and was also higher than W light, although the difference with W light was not statistically significant. This unexpected promotion of plant height under B light may be related to known photomorphogenic responses to B light under LED conditions, as discussed in the discussion. Moreover, although detailed morphological measurements (e.g., leaf area, thickness, or number of lateral shoots) were not conducted, visual and tactile observations indicated clear differences among light treatments (Fig. S1). Plants under W light were the shortest, with fewer leaves of moderate width but reduced thickness, a sparser canopy, and fewer lateral shoots. B light induced the tallest plants, featuring elongated internodes and a high number of small, narrow, and thin leaves (resulting in a limited overall leaf area) and modest lateral branching. RB light promoted the most compact growth, characterized by short internodes, thicker and larger leaves, a greater leaf number, and numerous lateral shoots. R light elicited relatively vigorous growth, with a good number of moderately thick and large leaves, intermediate plant height between that observed under RB and B light, and abundant vigorous lateral shoots.

The main effect of the BS treatment (Fig. 3B) showed that plant height increased significantly following BS application compared to the untreated condition (+5.6%).

Effect of light spectrum and BS on SRP and AJM production

SRP concentrations and yields

Figure 4 illustrates the main effect of BS treatment on SRP-related variables. In particular, leaf SRP concentration, as well as leaf, root, and total SRP yields, significantly increased following BS treatment compared to the untreated condition, with respective increases of 22.5% (Fig. 4A), 53.2% (Fig. 4B), 33.2% (Fig. 4C), and 34.4% (Fig. 4D).

AJM concentration and yield

Figure 5 illustrates the interaction between light spectrum and BS treatment on both AJM concentration (A) and yield (B). The highest AJM concentration was observed under the R:YES combination (298 ± 19.8 µg g^{-1} DW), while the lowest was recorded under the W:NO combination (122 ± 19.8 µg g^{-1} DW). Notably, the R:YES combination resulted in a significantly higher AJM concentration compared to all other treatment combinations (+144.3% compared to W:NO). Similarly, the highest AJM yield was highlighted under the R:YES combination (814.0 ± 82.9 µg plant^{-1}), whereas the lowest was observed under the R:NO combination (258.0 ± 82.9 µg plant^{-1}). This treatment significantly enhanced AJM yield compared to all other combinations (e.g., +138.0% compared to W:NO), except for RB:YES and B:YES, with which no significant differences were found. Furthermore, under

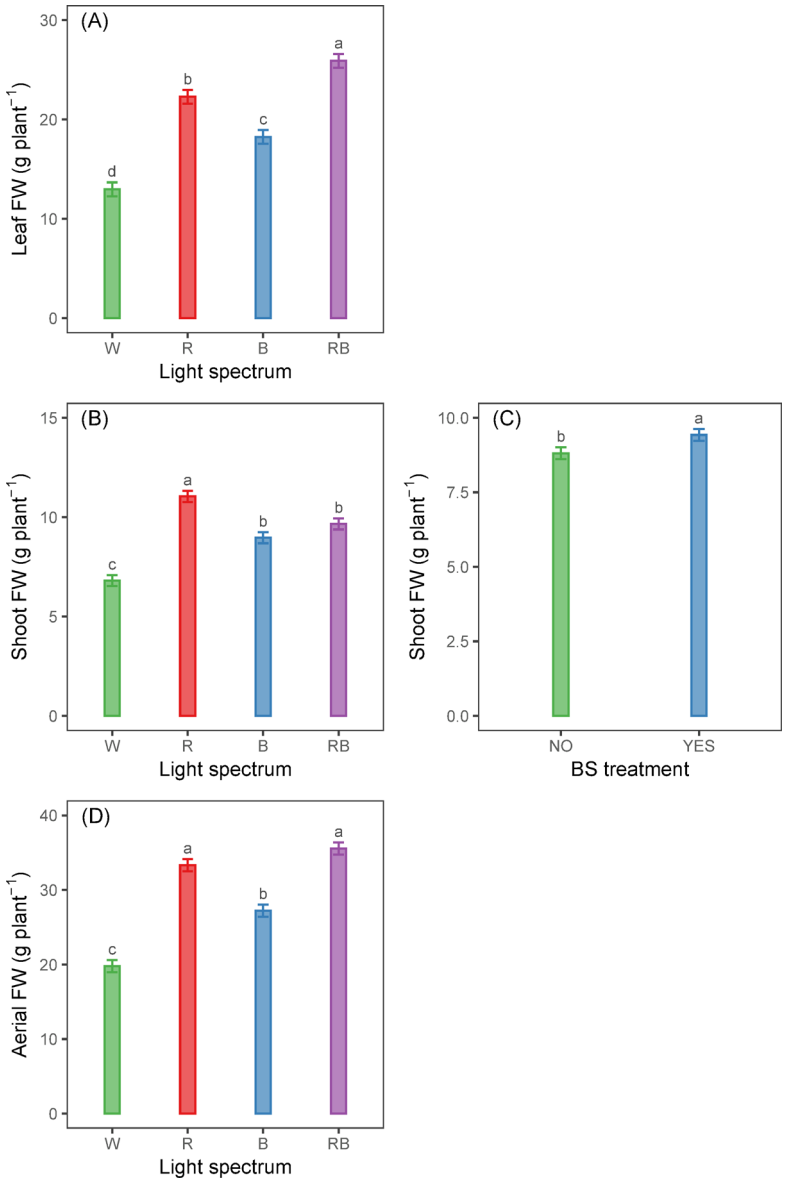

Fig. 1. Main effects of light spectrum and AMF-containing biostimulant (BS) on *C. roseus* fresh weights (FWs). (**A**, **B**, **D**) Leaf, shoot, and aerial (leaf + shoot) fresh weights under different light spectra: white light [W], red light [R], blue light [B], and a mixture of B and R light [RB]. (**C**) Shoot fresh weight under BS treatment (NO = untreated, YES = treated). Bars represent the standard error ($n = 7$). Means with different letters within each panel are significantly different at the 5% level (Tukey's adjustment for light spectrum, t-test for BS).

equal light spectrum conditions, the BS treatment generally showed a tendency to increase both the concentration and yield of AJM, as all light treatments combined with the BS exhibited higher AJM concentration and yield compared to those without the treatment. In contrast, under equal BS conditions, when considering both AJM concentration and yield, no specific light spectrum showed a clear tendency to outperform the others, with the sole and remarkable exception of the R:YES treatment.

Temporal dynamics in DWs, SRP, and AJM production

Table S1 summarizes the variations in DWs, concentrations, and yields across sampling times (T0 and T1). Between T0 and T1, all variables showed substantial increases, with the exception of leaf SRP concentration, which decreased. For instance, total SRP yield increased by 2277.1% and AJM yield rose by 716%.

Discussion

Absence of AMF colonization

The absence of mycorrhizal colonization across all samples in this study may be attributed to the high nutrient content of the soil mix (1200 g/m^3 soluble NPK, 1500 g/m^3 organic N, and micronutrients). In nutrient-rich

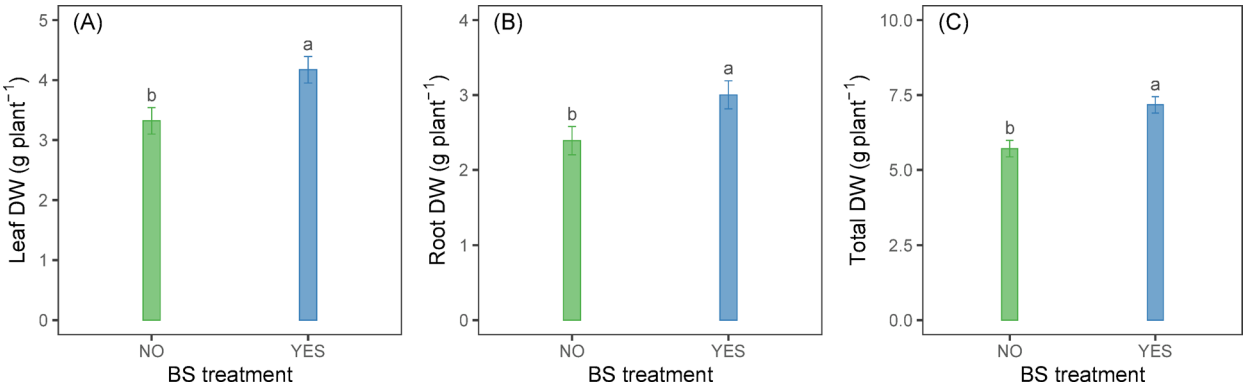

Fig. 2. Main effect of AMF-containing biostimulant (BS) on *C. roseus* dry weights (DWs). (**A**) Leaf, (**B**) root, and (**C**) total (leaf + root) DWs under BS treatment (NO = untreated, YES = treated). Bars represent the standard error ($n = 7$). Means with different letters within each panel are significantly different at the 5% level by t-test.

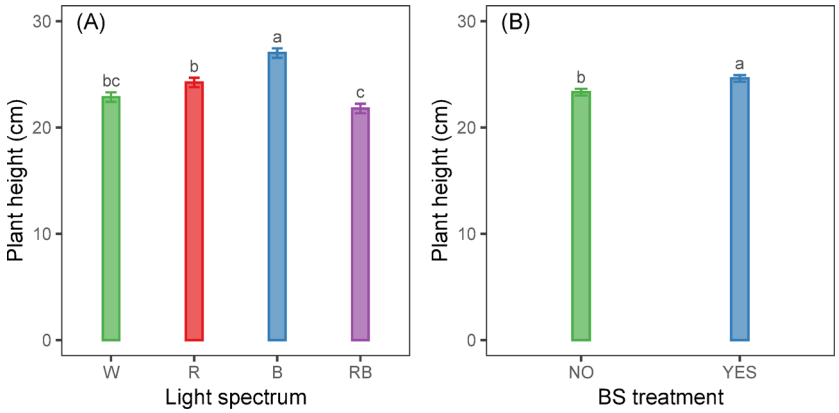

Fig. 3. Main effects of light spectrum and AMF-containing biostimulant (BS) on *C. roseus* plant height. (**A**), Light spectrum (white light [W], red light [R], blue light [B], and a mixture of B and R light [RB]), (**B**) BS treatment (NO = untreated, YES = treated). Bars represent the standard error (n = 7). Means with different letters within each panel are significantly different at the 5% level (Tukey's test for light spectrum, t-test for BS).

environments, especially those with high phosphorus availability (as was the case in our substrate), plants reduce their reliance on AMF, limiting or stopping the symbiosis as they can meet their nutritional needs independently[32].

Light spectrum effects

Plant growth

Light spectrum significantly influenced only plant FWs and plant height. Regarding FWs, at the organ level, both RB and R light significantly enhanced leaf and shoot FW, showing the most pronounced effects. However, RB light was more effective in increasing leaf FW, while R light had a stronger effect on shoot FW.

R and B light are the most efficiently absorbed wavelengths for plant photosynthesis. This is because the absorption spectra of chlorophylls and carotenoids peak mainly in the B (400–500 nm) and R (600–700 nm) regions [33]. R light, absorbed by phytochromes, supports the development of photosynthetic organs and has the highest quantum yield of CO_2 fixation[34,35]. It also promotes Rubisco production and enhances net photosynthetic rate (Pn). In turn, this contributes to increased aboveground biomass[35,36]. B light, mainly absorbed by cryptochromes and phototropins, enhances photomorphogenesis, stomatal development, opening and conductance [37,38]. It also increases chlorophyll a/b ratio, photosystem activity, and the expression of Calvin cycle–related genes compared to R light [39,40]. However, in general, monochromatic light can impair growth. R light can reduce plant biomass, leaf number, and chlorophyll content. It can also provoke excessive stem elongation ("R light syndrome"), due to lack of far-red (FR) light and suboptimal R:B balance[41]. On the other hand, B light can inhibit leaf expansion and root growth, and induce photoprotective responses, such as increased carotenoid accumulation[42]. In contrast, combining R and B light balances photoreceptor activation, and mitigates the adverse effects of monochromatic illumination. This results in enhanced plant growth and development, improving overall plant performance.

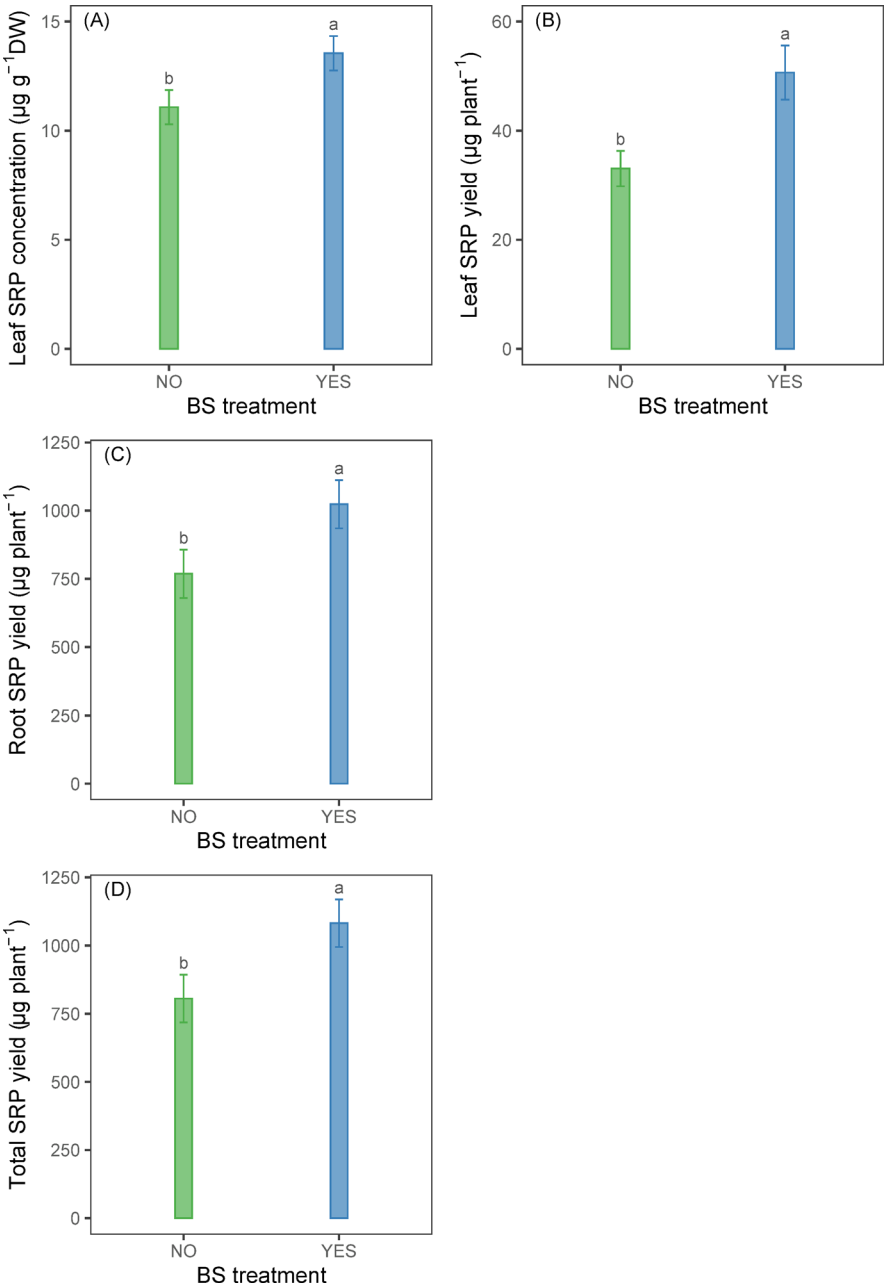

Fig. 4. Main effect of AMF-containing biostimulant (BS) on *C. roseus* serpentine (SRP) production. (**A**) Leaf SRP concentration, (**B**) leaf SRP yield, (**C**) root SRP yield, and (**D**) total (leaf + root) SRP yield under BS treatment (NO = untreated, YES = treated). Bars represent the standard error ($n = 7$). Means with different letters within each panel are significantly different at the 5% level by t-test.

Moreover, R and B light influence the synthesis and distribution of key plant hormones, such as auxins, cytokinins, and gibberellins, which regulate plant growth and development[43]. Auxins are primarily involved in cell elongation and regulate apical dominance, root initiation, and vascular differentiation[44]. Gibberellins promote cell growth in stems, leaves, and other aerial parts by inducing elongation and increasing internodal length[45]. Cytokinins regulate shoot meristem size, leaf primordia number, and the growth of leaves and shoots by stimulating cell division[46]. B light stimulates auxin production in various plant tissues, often linked to phototropic growth responses[47]. It can also enhance gibberellin levels through cross-talk between photoreceptors[48] (as better detailed below for the discussion on plant height). R light, on the other hand, can promote auxin and cytokinin biosynthesis through phytochrome-mediated regulation of genes involved in their biosynthesis and signalling pathways[49–51].

The significantly enhanced leaf FW observed under RB light can be mechanistically explained by the combined effects of photoreceptor activation and efficient excitation of photosynthetic pigments. Photosynthetic pigments, such as chlorophylls and carotenoids, absorb both R and B wavelengths effectively, leading to increased

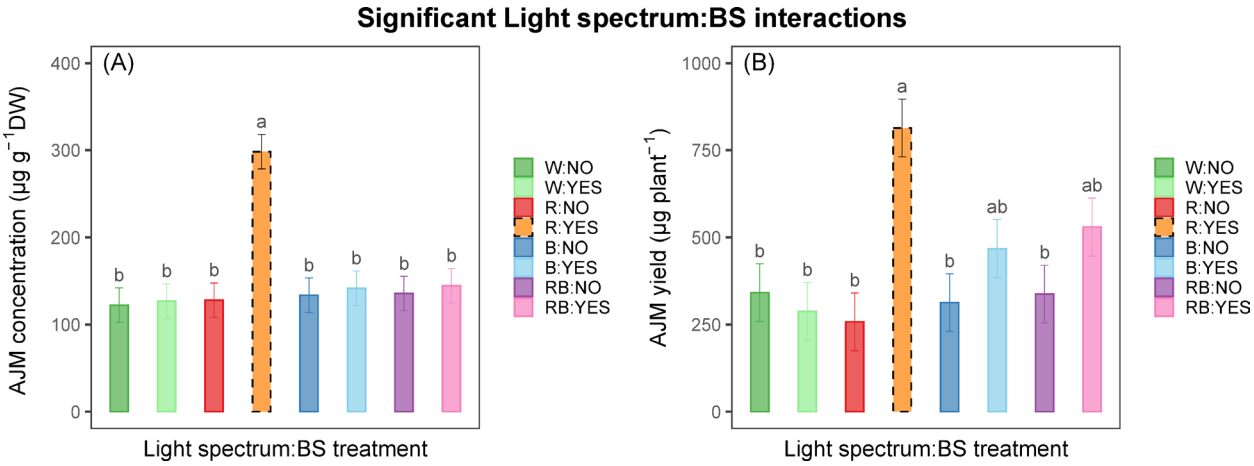

Fig. 5. Interaction between light spectrum and AMF-containing biostimulant (BS) treatment on *C. roseus* ajmalicine (AJM) production. (**A**) AJM concentration and (**B**) yield under all combinations of light spectrum (white light [W], red light [R], blue light [B], and a mixture of B and R light [RB]) and BS treatment (NO = untreated, YES = treated). The dashed border highlights the R:YES treatment to emphasize its distinctive response. Bars represent the standard error ($n = 7$). Means with different letters within each panel are significantly different at the 5% level by Tukey's adjustment.

photosynthetic activity and biomass accumulation. In parallel, the combination of R and B light activates multiple photoreceptors, including phytochromes, cryptochromes, and phototropins. These photoreceptors modulate the synthesis and distribution of growth-related hormones such as auxins and gibberellins, thereby promoting leaf development and contributing to enhanced leaf biomass production. These coordinated mechanisms therefore provide a clear mechanistic basis for our results. Nonetheless, earlier studies on *C. roseus* reported contrasting patterns, where R light was more effective than RB light. This inconsistency may arise from the distinct R:B ratios employed, underscoring the crop-specific importance of optimizing this parameter. For example, Fukuyama et al.[17] studied *C. roseus* "Titan" under different light spectra: W, R, B, and RB (R:B = 2:1). They observed the highest leaf FW under R light, followed by RB, W, and B. However, no significant differences were found between the R, RB, and W treatments.The contraast with the present study (where RB -R:B = 6:1- led to the highest leaf FW) may be due to the different R:B ratios used. Identifying the optimal R:B ratio is therefore critical for designing effective light spectra, as this ratio is highly crop-specific. For instance, Verma et al.[52] reported that in *Digitalis purpurea*, RB treatments (1:1, 8:2, 2:8) produced higher leaf FW than R, B, or W alone. Among these, RB 8:2 treatment was the most effective.

Similarly, the significant increase in shoot FW observed under R light can be attributed to the combined effects of enhanced chlorophyll excitation coupled with a phytochrome-mediated shift in hormonal balance. On one hand, increased chlorophyll excitation improves photosynthetic efficiency, supporting biomass accumulation. On the other, R light may have stimulated cytokinin biosynthesis more than that of auxin via phytochrome activation. This may have reduced the auxin:cytokinin ratio, thereby promoting meristematic activity and reducing apical dominance. These coordinated mechanisms support shoot development and lateral branching, explaining the observed increase in shoot FW. These results align with the findings of Fukuyama et al.[17], who also observed greater shoot FW under R light.

Finally, both RB and R light significantly increased total aerial FW, with RB showing a slightly stronger effect. Under RB light, the increase was primarily due to higher leaf FW, indicating a greater contribution of leaves. In contrast, under R light, the increase in aerial FW was mainly driven by higher shoot FW, suggesting a greater contribution of shoot tissues relative to leaves. The increased aerial FW under RB and R light may also be supported by visual and tactile observations, as highlighted in the results.

Concerning plant height, contrary to the common assumption that B light promotes plant compactness more effectively than R light[53], our results show that B light significantly increased plant height. This divergence may arise from earlier studies using broad-spectrum (non LED) lamps as a source of B light (e.g., metal halide and B-colored fluorescent lamps) alone or in the background of solar light, which complicates isolation of its specific effects[54]. In contrast, studies with monochromatic B light LEDs report genotype-, stage-, lighting-, and cultivation-dependent responses, with B light sometimes promoting elongation[55,56], and sometimes inhibiting it[57,58]. As reported by Kong et al.[48], one possible explanation for the elongation observed under B LEDs may lie in the spectral composition of those non-monochromatic LED sources and their impact on phytochrome activation. Many of those older light sources emitted small amounts of R and FR light, resulting in a high R/FR ratio that activates phytochromes, causing B light to exert a more suppressive effect on elongation than R light. [59,60]In contrast, B LEDs exhibit a lower phytochrome photostationary state (PPS)[61] compared, for example, with R light, which is associated with reduced phytochrome activation and may explain the enhanced elongation observed under B LED lighting[62]. Supporting this, Kong et al.[63] demonstrated that B light can induce a species-specific shade avoidance response (SAR) under low PPS conditions, as seen with pure B light or B light supplemented with minimal R/FR wavelengths. Under these conditions, B light acts as a shade signal via

multiple photoreceptors, including phytochromes (PHYs) and cryptochromes (CRYs). Furthermore, B light has been shown to increase levels of active gibberellins (GA_1, GA_4)[64]. This effect correlates with the upregulation of *GA20-oxidase* (a key enzyme in GA biosynthesis) as supported by higher expression of *PhGA20ox-1S* and *PhGA20ox-2L* genes[65]. Altogether, these findings suggest that under specific conditions, B light can promote elongation through a combination of altered photoreceptor activity and hormone-mediated signalling. Finally, while light intensity and photoperiod are known to interact with light quality (including B light) in modulating elongation responses[48], both were intentionally maintained constant in this study. This ensured that any observed effects could be attributed exclusively to the tested treatments (light spectrum and BS).

SRP and AJM production

SRP concentrations and yields were unaffected by light spectrum, either as a main effect or in interaction with BS. By contrast, AJM showed a different pattern: concentration was influenced both by light spectrum and by its interaction with BS (significant main effect of light spectrum and significant light spectrum:BS interaction), whereas yield was not affected by light spectrum alone (non-significant main effect) but was influenced by its interaction with BS (significant light spectrum:BS interaction). As previously explained, significant main effects were not analyzed for variables that also exhibited significant interactions, such as AJM concentration and yield, to avoid misleading conclusions.

Moreover, under equal BS conditions, differences among light spectrum treatments in both AJM concentration and yield were minimal, statistically non-significant, and showed no clear increasing trend for any particular spectrum, with the sole exception of R light combined with BS (R:YES).This treatment exhibited a dramatic and significant increase compared not only to the other light spectrum treatments coupled with BS, but also to all other combinations.

Consequently, the effects of light spectrum on AJM are discussed exclusively within the framework of the light spectrum:BS interaction (see "Synergistic Effects of R light and BS"), focusing on the dramatic and significant increase observed under R:YES.

BS effects

Before discussing the effects of the BS on plant growth and alkaloid production, it is important to note that AMF colonization did not occur in any of the treatments. Therefore, the observed effects cannot be attributed to direct mycorrhizal colonization. Although AMF were not detected in any root samples, we cannot entirely exclude the possibility of indirect effects mediated by non-colonizing AMF (e.g., early signalling events, shifts in the microbial community, viable but inactive spores, or residual metabolic activity). However, such interactions were beyond the scope of this study and were not investigated, and no morphological evidence of AMF presence was observed. Given the absence of colonization, it is more plausible that the observed plant responses were primarily driven by the bioadditive fraction of the BS. This fraction contains well-known biostimulant compounds that influence plant growth and metabolism (such as humic substances, keratin, and seaweed extracts – see Methods for full composition). Indirect AMF action, while not completely dismissible, is likely to have a negligible impact.

Plant growth

BS treatment had a significant effect on shoot FW. In general, the effects of biostimulants on increased biomass are commonly linked to enhanced photosynthetic efficiency[66]. It is well-established that biostimulants can either promote the biosynthesis or reduce the degradation of chlorophylls. This influences their concentration in the leaves and subsequently improves photosynthetic activity, electron transport in the photosystems, and overall biomass production[67]. Nofal et al.[27] investigated the effects of various biostimulants (humic acid, salicylic acid, and chitosan) on vegetative and rooting growth parameters, as well as on certain chemical constituents of a local *C. roseus* variety. They found that the FW of aerial plant parts significantly increased with the application of biostimulants compared to the control treatment.

BS application also significantly influenced leaf, root and total DW. Nofal et al.[27] observed that the application of biostimulants significantly increased the DWs of both the aerial plant parts and roots compared to the control treatment. Vernieri et al.[68] reported an increase in root dry mass following biostimulant treatments, potentially attributable to the presence of compounds such as humic substances and algal extracts that can exert hormone-like effects, thereby altering the plant's hormonal balance[69]. Specifically, the increase in root biomass may be linked to the action of auxin-like substances. Additionally, the significant increase in total DW observed with the BS treatment can be attributed almost equally to the increase in leaf and root DW. However, the leaf contribution is slightly greater than the root contribution.

Plant height was also significantly enhanced under BS treatment. Numerous studies in the literature highlight the ability of biostimulants to promote increased plant height. Notably, the study by Nofal et al.[27] on *C. roseus* demonstrated that all applied biostimulant treatments resulted in significantly greater plant height compared to the untreated condition. Several constituents of the BS (particularly humic substances and seaweed-derived extracts) are known to contain phytohormones or hormone-like compounds, such as auxins and gibberellins, These compounds, as previously noted, are key regulators of stem elongation through coordinated cell division and expansion. Their presence likely contributes to the observed stem elongation by modulating endogenous hormonal signalling pathways involved in shoot development.

SRP and AJM production

There are no published studies specifically investigating the effects of complex biostimulant formulations (or their interaction with LED light spectra) on SRP and AJM concentrations in *C. roseus*. In our study, BS significantly increased leaf SRP concentration and, under equal light conditions, also tended to enhance AJM levels. As discussed before, the bioadditive component of the BS used in this study contains several substances

that may influence alkaloid metabolism in *C. roseus,* including humic substances, seaweed extracts, and keratin. These compounds serve as rich organic nitrogen sources, supplying proteins, peptides, and amino acids such as tryptophan, tyrosine, and phenylalanine[66,70]. Since alkaloids are nitrogen-containing compounds, the availability of nitrogen is essential for their biosynthesis and accumulation in plants[71].

In *C. roseus*, TIA biosynthesis originates from the integration of the amino acid tryptophan (shikimate/indole pathway) and the monoterpenoid geraniol (MEP/iridoid pathway). These two precursors converge to form strictosidine, the central metabolite from which various TIAs, including AJM and SRP, are derived. AJM serves as the immediate precursor of SRP[72]. A detailed description of the biosynthetic steps leading to AJM and SRP, including the involvement of specific intermediates and enzymes such as tryptophan decarboxylase (TDC), geraniol 10-hydroxylase (G10H), strictosidine β-D-glucosidase (SGD), and heteroyohimbine synthase (HYS), is provided in the Supplementary Materials.

AJM and SRP predominantly accumulate in the roots of *C.roseus.* In contrast, leaves generally contain only small amounts of SRP and nearly undetectable levels of AJM[6,73]. In root tissues, a major portion of AJM is oxidized into SRP by vacuolar peroxidases (*PRX*)[74], while the remainder appears to remain sequestered within vacuoles[74]. More recently, a cytochrome P450 enzyme named serpentine synthase (*CrSS*) was identified in leaf tissues and shown to catalyze the conversion of AJM to SRP[75]. *CrSS* localizes to the endoplasmic reticulum of idioblast and laticifer cells and plays a key role in foliar SRP biosynthesis. Silencing *CrSS* via virus-induced gene silencing (*VIGS*) in *C. roseus* leaves resulted in a marked accumulation of AJM and a concomitant decrease in SRP, confirming that AJM is the immediate biosynthetic precursor of SRP in leaf tissues[75]. In wild-type plants, AJM is therefore rapidly converted to SRP by *CrSS*, leading to near-complete turnover and explaining the very low or undetectable levels of AJM in leaves (consistent with our own finding of no detectable AJM in leaf extracts). These data may also support the hypothesis that AJM is translocated via the phloem from roots to leaves, where *CrSS* catalyzes its conversion to SRP[75,76]. Although it is plausible that root-derived AJM is translocated to the aerial tissues via the phloem, no definitive evidence currently establishes the route or directionality of this systemic transport[77]. Moreover, while TIAs require specific transporters for intercellular and intertissue movement, only a few transporters involved in the TIA pathway have been characterized to date, and the majority remain unidentified[78]. The spatial compartmentalization and tissue-specific enzyme expression of SRP and AJM likely explain their differential accumulation across organs and their distinct responses to light spectra and biostimulants.

Furthermore, studies have demonstrated that supplementing cultures with precursor molecules, including tryptophan and phenylalanine, can enhance alkaloid accumulation in *C. roseus* cell suspensions and hairy root cultures[79,80]. Notably, administering tryptophan at specific developmental stages can mimic auxin-like effects, thereby promoting the metabolic flux toward indole alkaloid biosynthesis.[80] Humic substances and seaweed extracts also contain phytohormones or phytohormone-like compounds, such as auxins and cytokinins[81]. The application of auxin has been reported to stimulate *TDC* activity in *C. roseus* seedlings [82], while cytokinin enhances the expression of *G10H* gene[83].Additionally, Nofal et al.[27] reported increased total alkaloid concentrations in *C. roseus* leaves treated with various biostimulants, including salicylic acid, humic acids, and chitosan, compared to the untreated control.

These combined biochemical and regulatory factors provide a coherent mechanistic explanation for the observed increase in SRP concentration in leaves following BS application. Primarily, the BS likely enhances the biosynthesis of AJM in the roots by supplying nitrogen-rich amino acid precursors (e.g., tryptophan) and hormone-like signaling molecules (e.g., auxins, cytokinins). These compounds are known to upregulate key upstream enzymes in the TIAs biosynthetic pathway, such as *TDC* and *G10H*. The increased production of AJM in the roots may then be transported via the phloem to the leaves, where it serves as a substrate for local SRP biosynthesis. Simultaneously, the BS may exert a direct effect on leaf tissues by enhancing the expression and the activity of *CrSS*, which catalyzes the conversion of AJM to SRP within the endoplasmic reticulum of leaf cells. The combined increase in AJM substrate availability and potentially upregulated CrSS activity in leaves can synergistically lead to higher leaf SRP accumulation.

In summary, BS treatment appears to promote SRP synthesis in leaves through a dual mechanism: indirectly by augmenting root-derived AJM biosynthesis and its subsequent transport to leaves, and directly by modulating enzymatic conversion processes localized in leaf tissues.

BS treatment also significantly enhanced SRP yields. Regarding the leaves, both leaf DW and leaf SRP concentration contributed significantly to the observed increase in SRP yield. However, the leaf DW showed a predominant role. This indicates that the BS primarily enhanced leaf biomass, which in turn facilitated greater SRP yield. For the roots, the increase in yield following BS application can be attributed to the significant effect of DW, as the BS did not significantly affect the root SRP concentration. This suggests that BS promoted root growth and biomass accumulation, likely by enhancing nutrient uptake rather than directly increasing SRP content. The increased biomass thus contributed to the higher root yield.

Regarding total SRP yield, although both leaf and root yields significantly contributed to the overall increase, the increase in leaf yield had the greatest impact. However, it is important to note that in *C. roseus*, roots typically produce greater amounts of SRP compared to leaves, as confirmed in our study (leaves, without BS: 11.1 μg g^{-1} DW, with BS: 13.6 μg g^{-1} DW; roots, without BS: 318 μg g^{-1} DW, with BS: 332 μg g^{-1} DW). Given the similar DWs between leaves and roots in our study, this resulted in significantly higher yields in roots (without BS: 769 μg $plant^{-1}$, with BS: 1024 μg $plant^{-1}$) compared to leaves (without BS: 33.1 μg $plant^{-1}$, with BS: 50.7 μg $plant^{-1}$). Therefore, the greater contribution of leaf yield to the total SRP yield in this case may reflect the synergistic effect of improved leaf biomass on overall plant productivity, rather than a direct comparison of leaf versus root SRP production. This underscores the importance of optimizing both leaf and root growth for maximizing total SRP yield in *C. roseus*, as both tissues contribute significantly to alkaloid production.

Moreover, the overall tendency for increased AJM yield under the sole BS treatment can be attributed to enhanced root biomass rather than higher AJM concentration. Indeed, the BS significantly increased root biomass, whereas differences in AJM concentration between light treatments with and without the BS were minimal and not significant (except for R:YES).

Taken together, these results indicate that the primary effect of BS in *C. roseus* was to enhance leaf and root biomass, which in turn increased total SRP and AJM yields. This suggests that, under the sole effect of BS, improvements in plant growth were the main driver of higher alkaloid production, rather than direct changes in their concentrations.

Synergistic effects of R light and BS

The empirical results of the present study show that the interaction between light spectrum and BS significantly affected only AJM, with the R:YES combination causing a significant increase in both its concentration and yield. Regarding AJM concentration, to interpret these empirical results, we propose a potential regulatory mechanism for its increase under R:YES**.** This dramatic and significant enhancement provides new insights into the complex interplay between light quality and biostimulants in regulating alkaloid biosynthesis. However, although the direct effects of LED light spectrum and biostimulants on AJM synthesis remain unexplored in the literature, several lines of evidence may support the plausibility of our results. In addition to the previously discussed effects of biostimulants on alkaloids, R light is recognized as an important regulator of their biosynthesis, primarily through its dependence on phytochrome-mediated signalling pathways, secondary messengers (G protein and CaM)[84,85], and the expression of key biosynthetic genes[86]. In *C. roseus*, R light promoted VDL biosynthesis by increasing the expression of the transcription factor gene *GATA1* and VDL pathway genes tabersonine-16-hydroxylase2 (*T16H2*), tabersonine-3-oxygenase (*T3O*), tabersonine-3-reductase (*T3R*), desacetoxyvindoline-4-hydroxylase (*D4H*), and deacetylvindoline-4-O-acetyltransferase (*DAT*). Previous studies have shown that red light (PPFD 150 μmol m^{-2} s^{-1}) generally enhances VDL and CAT concentrations and yields in *C. roseus* leaves compared to other spectra. Ohashi-Kaneko et al.[19,20] reported that red LEDs were more effective than white fluorescent lamps for increasing these alkaloids. Similarly, Quadri et al.[14] confirmed the promotive effect of red light at this intensity over white light, while Fukuyama et al.[17] observed higher VDL and CAT yields under red light than under blue, mixed red/blue, or white light at the same PPFD.

Although direct evidence for R light involvement in AJM is currently lacking, it is plausible that similar phytochrome-mediated regulatory mechanisms may operate in the AJM branch, potentially upregulating TIA midstream biosynthetic genes such as *SGD* and *HYS*[72].

Building on these literature-based observations, a plausible mechanistic explanation for the enhanced AJM levels under R:YES involves the integration of light-induced signalling and biostimulant-mediated stress responses. R light, likely through phytochrome-mediated signalling, strongly primes the expression of midstream genes in the TIA biosynthetic pathway. Concurrently, BS supplies nitrogenous precursors to fuel the pathway, and hormones or hormone-like compounds that actively upregulate upstream TIA genes, thereby boosting the overall TIA flux and promoting AJM accumulation. This proposed mechanism is speculative and inferred from analogies with other TIA branches, but remains to be experimentally confirmed. This synergy could manifest as a more pronounced increase in AJM levels under R:YES treatment. Moreover, the absence of similar effects with the other light spectra highlights a specific role of R light in enhancing the efficacy of BS in this context.

Regarding AJM yield, the following explanation is based directly on empirical data. The significant increase observed with R:YES is primarily due to higher AJM concentration rather than changes in root DW, as no significant interaction between light and BS was found for root DW. This explanation directly relies on measured root DW and AJM concentration to account for the observed yield. Such a targeted effect is especially advantageous, as increasing AJM yield through greater root biomass would require larger plants, occupying more space and potentially diluting AJM concentration per unit biomass. In contrast, enhancing AJM levels without substantially increasing biomass offers a more efficient strategy, conserving both space and resources. This is especially advantageous for agricultural or biotechnological applications where maximizing the metabolite yield is more critical than simply increasing root size and biomass.

Differential regulation of AJM and SRP by light spectrum and BS

Our findings reveal a differential regulation of AJM and SRP biosynthesis in *C. roseus* under the combined influence of light spectrum and BS application. The strong stimulation of AJM accumulation under R light in the presence of BS suggests that environmental cues interact with chemical elicitors to modulate upstream and midstream steps of the TIA pathway. This interaction may involve phytochrome-mediated signalling cascades that enhance precursor availability in roots, thereby driving AJM biosynthesis. Such light–elicitor synergism has been reported in other plant systems[87], supporting the idea that spectral quality can modulate the effectiveness of biostimulants in secondary metabolism. Interestingly, BS alone promoted AJM availability for phloem transport and subsequent conversion into SRP in leaves, but appeared to repress AJM-to-SRP conversion in roots, likely through downregulation of PRX activity. This dual effect highlights the organ-specificity of biostimulant action, a phenomenon also observed in other alkaloid-producing species[30,88]. By contrast, SRP formation was largely independent of the light spectrum and instead associated with BS-induced activation of CrSS in leaf tissues. The observation that total SRP yield correlated more strongly with leaf biomass than with SRP concentration, whereas AJM yield was primarily driven by AJM concentration rather than root biomass, further underscores the distinct contributions of growth- and concentration-dependent factors in shaping metabolite output.

Taken together, these results suggest a dual-mode model of BS action: (i) in roots, BS synergizes with R light to drive AJM accumulation, while simultaneously limiting its conversion to SRP, with AJM yield primarily driven by AJM biosynthesis; (ii) in leaves, BS stimulates SRP biosynthesis regardless of light conditions, with yield strongly linked to biomass accumulation. From a broader perspective, these findings highlight the potential of

integrating light quality manipulation with targeted biostimulant application to optimize specific alkaloid yields in *C. roseus*.

Temporal dynamics effects

From T0 to T1, both leaf and root DW naturally increased due to plant growth, with leaves contributing more to the total DW gain. In contrast, SRP concentrations exhibited opposite trends in the two organs: decreasing in leaves and increasing in roots. All precise DW and concentration values, as well as derived yields and their relative percentage variations, are reported in Table S1. Our findings seem to be in agreement with existing literature. For instance, Iwase et al.[89] reported that the SRP concentration in *C. roseus* leaf cultures rose until day 12 of cultivation, then declined, whereas Li et al.[90] found that SRP steadily increased in hairy roots over 30 days. Consequently, higher leaf SRP yield at T1 (47.6 µg plant^{-1} vs 18.2 µg plant^{-1} at T0; + 161.5%), results from increased leaf biomass, while increased root SRP yield (T0: 21.4 ± 3.8 µg plant^{-1} vs T1: 896.0 ± 66.5 µg plant^{-1}; + 4086.9%) is mainly driven by higher SRP concentration.

Similarly, the improvement in AJM concentration from T0 to T1 (50.9 ± 4.3 µg g^{-1} DW vs 153.9 ± 9.9 µg g^{-1} DW; + 202.4%) aligns with findings from previous studies. For instance, Jaleel et al.[91] reported a marked increase in AJM content during the growth of two *C. roseus* varieties. The observed increase in AJM yield at T1 (418.6 ± 35.9 µg plant^{-1} vs 51.3 ± 4.5 µg plant^{-1} at T0) is attributed to both the increased root DW and AJM concentration, with the latter contributing more substantially.

The contrasting temporal trajectories of SRP accumulation in leaves and roots can be mechanistically interpreted in the light of organ-specific growth dynamics and compartmentalized regulation of the TIA pathway. In leaves, the observed decline in SRP concentration over time may be explained by a dilution effect associated with the rapid expansion of foliar biomass. This is consistent with the notion that SRP biosynthesis in leaves, mediated predominantly by CrSS, remains active but is outpaced by the increase in dry matter, resulting in lower concentrations despite ongoing metabolic activity. By contrast, roots exhibited a marked rise in SRP concentration, even as root dry mass increased substantially. This may reflect an enhanced metabolic flux from AJM to SRP, catalyzed mainly by vacuolar PRX, resulting in net accumulation that exceeds growth-related dilution. Taken together, these patterns, although they remain to be experimentally confirmed, suggest that while leaf SRP dynamics are largely shaped by growth-driven dilution of a relatively stable biosynthetic flux, root SRP dynamics reflect a genuine upregulation of local biosynthesis and conversion.

Overall, these temporal patterns suggest that harvesting at T1 (92 DAP) represents a practical and effective timepoint for maximizing both biomass accumulation and alkaloid yield in *C. roseus*, providing a clear guideline for growers aiming to optimize production.

Conclusions

Our findings demonstrate that in *C. roseus* cultivated under indoor condition, the application of R light combined with BS over a 92-day treatment period (T1), following a 60-day pre-treatment under W light, represents the most effective strategy to optimize AJM and SRP production, while ensuring vigorous yet size-controlled plant growth and potentially reducing energy costs. Specifically, SRP and AJM yields increased markedly with plant age, indicating that harvesting at T1 is the optimal time point to maximize the accumulation of both metabolites. While BS treatment alone is sufficient to significantly enhance and thus optimize SRP yields, its combination with R light is specifically required to significantly improve both the concentration and yield of AJM, maximizing their levels. Moreover, adding R light is advantageous even when the primary goal is maximizing SRP yields, as it enhances biomass accumulation and counteracts the BS-induced increase in plant height, thereby improving size control, a key benefit in space-limited indoor systems. R light also offers the highest photosynthetic photon efficacy (PPE), making it an energy-efficient and cost-effective solution in controlled environments.

Despite these promising results, some limitations should be acknowledged. Our understanding of the interactions between LED light spectra and BS effects remains incomplete. Future studies should incorporate transcriptomic and gene expression analyses to elucidate the underlying molecular mechanisms, while also assessing the long-term impacts of light spectrum–BS combined treatments. In particular, such analyses should focus on the specific signalling components regulating AJM under R light combined with BS treatments, and SRP under BS treatment alone, while evaluating whether the observed responses extend beyond controlled indoor conditions to field applications. Expanding the research to include additional light spectra (e.g., FR, UV), varying BS doses, and a broader range of *C. roseus* cultivars will be essential for further optimizing integrated cultivation protocols aimed at maximizing AJM and SRP yields. The unexpected absence of mycorrhizal colonization also warrants further investigation. Future research should explore different AMF species, assess potential genotype-specific responses, and optimize substrate composition (particularly by reducing phosphorus availability) to better understand and improve the conditions necessary for successful symbiotic establishment.

Methods

Plant material and growing conditions

Seedlings of *C.roseus* "Titan Really Red," approximately 40 days old and exhibiting 2–3 pairs of leaves, were sourced from a large commercial nursery. These seedlings were provided on October 5, 2023 in specialized cell trays. On the same day, they were promptly transplanted into 1.4 L square plastic pots (dimensions: 10 × 10 cm; height: 17 cm; Bamaplast, Pistoia, Italy), filled with a professional soil mix (VIV COCCO TIIRENO 2, Vigorplant, Lodi, Italy). The plants were then placed in an environmentally controlled growth room for a 60-day pre-treatment acclimatation period, where they were maintained under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of approximately 100 µmol m^{-2} s^{-1}, provided by white LED lights (Extended White Combo 150 W, 3221 K; C-LED, Imola, Italy). The room temperature was set to 23 °C. PPFD was measured

at the top of the canopy using an LP471/PAR quantum radiometric sensor (Delta Ohm S.r.l., Padua, Italy). The pre-treatment phase was necessary to allow the young seedlings to acclimatate to the new environment and recover from transplant stress. This phase also served to avoid exposing them immediately to potentially stressful experimental treatments (such as monochromatic light spectra), to standardize their growth conditions after possible variability in the nursery phase, and to ensure they reached a sufficient level of biomass and vigor before the start of the actual experimental treatments. Throughout the pretreatment period, plants were irrigated with tap water, adjusted to a pH of 6 using 38% nitric acid, as required. At the end of the 60-day period, plants in good health and uniform in size were selected and subsequently assigned to their respective treatment groups.

Experimental treatments

The experiment involved eight treatments, obtained by combining four LED light spectra with two AMF-containing biostimulant (BS) conditions, resulting in a total of eight treatment combinations (Table 2, Fig. S2).

Plants were exposed to experimental treatments on December 4, 2023. The light treatments were conducted in the same growth room used during the pretreatment acclimation period. The chamber conditions were maintained as follows: a 16/8-h photoperiod, a temperature of 23 °C, and relative humidity of 70%. Canopy-level PPFD was measured using the same quantum radiometric sensor employed during the pretreatment acclimation period (LP471/PAR, Delta Ohm S.r.l., Padua, Italy). The PPFD of 150 µmol m^{-2} s^{-1} was selected based on the results of Fukuyama et al.[10], who observed that this PPFD maximized concentrations of VDL and CAT, two alkaloids within the same metabolic pathway (TIAs) as AJM and SRP.

A commercial biostimulant containing AMF was used to inoculate the plants belonging to BS treatments. This commercial inoculum was chosen over a laboratory-prepared alternative as it more accurately represents the practical application of AMF in medicinal plant cultivation, particularly in terms of inoculum quantity and quality. The biostimulant composition included:

a. *Bioactive particles* (colonized root fragments, spores, and mycelium fragments) from five AMF species (*Claroideoglomus etunicatum, Claroideoglomus claroideum, Rhizophagus irregularis, Funneliformis geosporus, Funneliformis mosseae*), with a minimum infective propagule count of 200,000 per kg and an average of 325,000 propagules per kg, carried on an inert substrate composed of zeolite (390 g per kg; green particles, size fraction 0.5–2.5 mm) and expanded clay (500 g per kg; brown particles, size fraction 1–2.5 mm);
b. *Bioadditive components* (52 g per kg of product), comprising natural minerals (dolomite, patentkali, milled phosphates), seaweed extracts (alginates), natural keratin, humates, and water-storing polymer granules.

Plants chosen for BS treatment were randomly selected and inoculated immediately prior to subjecting them to the light treatment. A 40-g dose of the product was applied to each pot (40 g per 1.4 L of substrate per pot) by distributing the dose into four 10-cm deep cavities, with 10 g per cavity, positioned directly beneath the transplanted seedlings.

Sampling procedures

Leaf and root samples were collected to assess dry weights and the concentrations of SRP and AJM. Moreover, root samples were harvested to investigate mycorrhizal presence. To monitor temporal changes in dry weights, concentrations, yields, and to evaluate mycorrhizal colonization, plants were sampled at two distinct time points: T0 (immediately prior to the application of BS and LED light treatments) and T1 (92 days after the pre-treatment -DAP-, corresponding to a total 152 days of cultivation). At T0, five plants were randomly selected for alkaloid analysis and mycorrhizal colonization assessment. At T1, seven plants per treatment group were sampled for alkaloid analysis, and three additional plants per treatment group were examined for AMF colonization.

Arbuscular mycorrhizal colonization assessment

Six portions of fine roots were randomly sampled from the entire root system of each plant and processed according to Vierheilig et al. [92] for endomycorrhizae visualization. Briefly, the roots were decolorized in a 10% KOH solution at 120 °C for 35 min. Following decolorization, the samples were rinsed with distilled water to remove any residual KOH. The roots were then stained for 10 min at 90 °C in a 5% acetic acid solution

T[a]	S[b]	PPFD[c]	LS[d]	BST[e]	TTC[f]
W (control)	LED extended white Combo 150 W (C-LED)	150	White light (W; R = 49,6%, G = 35.5%, B = 13.2%)	NO	W:NO
W + BS				YES	W:YES
R	LED red combo 150 W (C-LED)		Red light (R, 658 nm)	NO	R:NO
R + BS				YES	R:YES
B	LED blue toplight 150 W (C-LED)		Blue light (B, 446 nm)	NO	B:NO
B + BS				YES	B:YES
RB	LED Hortis Toplight 150 W (C-LED)		Mixture of R and B (6:1)	NO	RB:NO
RB + BS				YES	RB:YES

Table 2. Experimental treatments and total treatment combinations. [a]T:Experimental treatments, [b]S: Light source, [c]PPFD: photosynthetic photon flux density (µmol m^{-2} s^{-1}), [d]LS: Light spectrum, [e]BST: AFM-containing biostimulant treatment, [f]TTC: total treatment combinations.

Compound	Parent ion (*m/z*)	Product ions (*m/z*)[a]	Cone voltage (V)	Collision energy (eV)
AJM	353.4	144.4, *209.6*	25	30
SRP	349.4	317.5, *227.3*	35	35
IS (vindoline-d3)	460.5	430.4, *400.2*	35	40

Table 3. Analyte-dependent MRM MS parameters. [a]In italic, qualifier ions.

containing 5% 'Blue Pelikan' ink. After a final rinse to remove excess stain, the stained roots were mounted on microscope slides for subsequent microscopic analysis.

HPLC–MS/MS analysis for serpentine and ajmalicine determination

Quantitative analysis of SRP and AJM was carried out exploiting high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS/MS). The analytical method used was based on a previously validated one[14], applying tailored modifications to optimize detection and quantitation for SRP and AJM, under the specific conditions of this study. These adaptations ensured accurate and precise analysis of the target alkaloids, while maintaining consistency with the original analytical instrumental approach.

The chromatographic separation was achieved using a Waters Alliance e2695 system (Waters Corporation, Milford, MA, USA) paired with a 2998 photodiode array detector and a Quattro Micro triple-quadrupole mass spectrometer. This configuration, operating in electrospray ionization positive mode (ESI +), was controlled via MassLynx 4.1 software. For the chromatographic separation, a Waters XTerra MS C18 column (100 × 2.1 mm, 3.5 µm particle size; Waters Corporation, Milford, MA, USA) was used, along with a matching guard column. The mobile phase comprised 0.15% formic acid in water (component A) and 0.15% formic acid in acetonitrile (component B). The gradient elution started at an A:B ratio of 90:10, maintained for 1 min, followed by a change to 70:30 over 1 min (then held for 3 min), further transitioning to 50:50 over 1 min (held for 3 min), and concluding with a return to the initial ratio within 1 min, including a re-equilibration step. This gradient was originally designed to ensure optimal separation of SRP and AJM, while avoiding potential carryover effects from prior analyses.

For the MS and MS/MS detection, multiple reaction monitoring (MRM) in ESI + mode was employed. Parameters for ionization and fragmentation were optimized by direct infusion of standard solutions of SRP and AJM (1 µg/mL in methanol). Key settings included a source voltage of 3.5 kV, a source temperature of 125 °C, and a desolvation temperature of 380 °C, with nitrogen as the desolvation gas and argon as the collision gas. Precursor and product ions, along with cone voltages and collision energies, were refined for the target analytes (Table 3). To ensure reliable analyte quantitation under these conditions, key validation parameters were assessed in terms of linearity, sensitivity, precision and internal standard (IS) suitability. The analytical method demonstrated a limit of detection (LOD) of 0.1 µg g^{-1} DW for SRP and 0.2 µg g^{-1} DW for AJM. Additionally, the limit of quantification (LOQ) was established at 0.3 µg g^{-1} DW for SRP and 0.6 µg g^{-1} DW for AJM. Linearity was assessed over the range 1–1000 µg g^{-1} DW using matrix-matched standards, yielding coefficients of determination (R^2) greater than 0.9990 for both analytes. Intra-day precision, expressed as relative standard deviation (%RSD), was below 6% for both analytes across the calibration range. Vindoline-d_3 was used as IS at a final concentration of 1 µg g^{-1} DW, selected for its structural similarity and comparable chromatographic and ionization behavior, ensuring accurate signal normalization.

Sample preparation protocol

Plant materials were air-dried at 40 °C to achieve constant weight, followed by fine grinding by means of an electric analytical mill. A 0.25 g sample of powdered material was extracted in 2.5 mL of 70% ethanol, under continuous agitation for 24 h. This process was repeated three times, and the extracts were pooled, filtered and subjected to vacuum evaporation at 40 °C using a vacuum-assisted rotary concentrator/evaporator. The resulting extracts were then dissolved in methanol (to reach a theoretical concentration of 1 mg/mL), centrifuged and filtered through nylon filters.

The workflow was established through comprehensive sample preparation protocol optimization, i.e. hydroalcoholic mixtures based on both ethanol and methanol in the 30–90% alcohol percentage were screened, along with different extraction times, repetition cycles, and agitation conditions. The selected configuration, consisting of three 24-h extractions under continuous agitation with 70% ethanol, provided optimal recovery of the alkaloid fraction and ensured compatibility with the following clean-up protocol. For enhanced selectivity and sensitivity in downstream analysis, a microextraction by packed sorbent (MEPS) procedure was applied for sample cleanup. Using a BIN (barrel insert and needle) with a mixed-mode C8 + SCX sorbent, the extracts were sequentially activated, equilibrated and processed with methanol and ultrapure water. After a washing step with a water–methanol mixture (90:10), the analytes were eluted with methanol. The eluate was vacuum-dried, resuspended in a 70:30 mixture of components A and B of the HPLC system mobile phase, and subjected to the HPLC–MS/MS analysis.

Experimental design and statistical analysis

A factorial experimental design (4 × 2) was used to investigate the effects of two factors on various traits of *C. roseus*: light spectrum (four levels: "W", "R", "B", "RB") and BS treatment (two levels: "NO", "YES"). This resulted in eight treatments, with seven plant replicates per treatment. Plants were randomly assigned to treatments within the controlled growth chamber, with each treatment placed on a separate shelf. Randomization was

performed using the R DiGGer package, which utilizes the Mersenne Twister pseudo-random number generator, in a manner consistent with that used by Brien et al.[94]. Environmental conditions were carefully regulated, and preliminary uniformity tests confirmed that they were homogeneous across all experimental units, with no significant differences observed between the shelving units of the growth chamber.

Regarding the tests, the following procedures were carried out to ensure the uniformity of environmental conditions:

- Verification of uniform light distribution across all shelf levels was conducted in two phases: first, a preliminary assessment without plants was performed using the Dialux lighting design software (DIAL GmbH, Lüdenscheid, Nordrhein-Westfalen, Germany), to model and optimize the light distribution pattern based on fixture positioning and chamber geometry; subsequently, in situ measurements were carried out with plants present inside the growth chamber, using the quantum sensors previously described, in order to verify and adjust the lamp intensity to achieve a consistent PPFD of 150 µmol m^{-2} s^{-1} at canopy height;
- Evaluation of temperature stability both at chamber level and at each shelf, alongside assessment of potential heat emission from the LED fixtures, which confirmed that the heat produced was negligible and did not cause measurable temperature variation (target chamber temperature: 23 °C);
- Assessment of temperature and relative humidity uniformity across all shelf positions, performed using the methodology proposed by Barbaresi et al.[95], later refined for indoor conditions in a subsequent work[95], as detailed below.

The approach of Barbaresi et al.[94,95] compares sensor data from different locations (two T and rH sensors for each shelf) within the chamber to two reference thresholds:

- The Identity Reference Value (IRV), based on the resolution and accuracy of the sensors, which defines when two measurements can be considered identical;
- The Acceptable Reference Value (ARV), defined based on the performance of the environmental control system and the specific goals of the experiment, which sets the maximum allowable difference between environmental zones for them to be considered equivalent.

The thresholds used in this study were:

- IRV: Temperature: 0.7 °C; Humidity: 4.7%
- ARV: Temperature: 1.5 °C; Humidity: 5%

It is important to note that these ARVs are substantially more stringent than the environmental variability typically observed in the natural habitats of *C. roseus*.

According to the methodology described in the cited works, temperature and humidity data were recorded every three minutes for each treatment. The recorded data were cleaned and analyzed as follows:

- For each timepoint, the differences between the environmental parameters of each treatment pair were calculated;
- For each sensor pair (i, j), the following index was computed:

$$z_{ij} = |m_{ij}| + sd_{ij}$$

  where *m* is the mean and *sd* is the standard deviation of the differences over time;
- The resulting *z*-values were then compared with the IRV and ARV thresholds.

All treatments were considered to be under equivalent environmental conditions if, for both temperature and humidity, all *z*-values remained below the ARV.

The following traits of *C. roseus* were therefore analyzed to assess treatment effects:

1. Leaf, shoot and aerial (leaf + shoot) fresh weight (FW) of the plant.
2. Leaf, root, and total (leaf + root) dry weight (DW) of the plant.
3. Plant height
4. Leaf and root concentrations and yields of SRP and AJM.
5. Mean SRP and AJM concentrations in the plant.
6. Total SRP and AJM yields in the plant.

To calculate yields (µg $plant^{-1}$), the DW (g $plant^{-1}$) of leaves or roots was multiplied by the concentration (µg g^{-1} DW) of the respective tissues. The total SRP/AJM yield (µg $plant^{-1}$) was determined by summing the SRP/AJM leaf yield and the SRP/AJM root yield. The mean concentration of SRP or AJM in the plant was obtained by weighting the concentrations in leaves and roots according to their DWs.

Data at "T0" (before treatment) were analyzed by calculating the mean of yields, concentrations, and dry weights from five sampled plants.

The statistical analysis for this study was performed using R Studio version 4.3.3. Linear models (lm) were used to evaluate the effects of 'Light' and 'BS', and their interaction on the dependent variables. Normality and homogeneity of variances were checked using quantile–quantile and residual plots, respectively. As leaf SRP yield did not meet these assumptions, it was log-transformed before analysis.

P-values were obtained using the Anova function (Type III) from the car package[96]. Estimated marginal means (EMMs) were calculated using the emmeans package [97] to calculate estimated marginal means (EMMs), and for leaf SRP yield, EMMs were back-transformed to the original scale for interpretation using the type = "response" argument. Pairwise comparisons were conducted using the Tukey adjustment applied via the multcomp::cld function in the multcomp package[98]. Simple pairwise comparisons (involving only two means) were performed using a T-test through the cld function for grouping. All statistical analyses were performed at a significance level of 0.05. Data visualization was carried out with the ggplot function from the ggplot2 package[99], and results are presented as means ± SE.

Data availability

The datasets generated during the current study are available in the Figshare repository, [https://figshare.com/s/5ab602ba900fd1bca3cb] (https:/figshare.com/s/5ab602ba900fd1bca3cb)

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Author contributions

A.Q. conceived and designed the study, conducted the experiments and data collection, performed statistical analyses using R, prepared the figures, and wrote the manuscript. B.S., M.T. contributed to the experiments and data collection. A.Z. and P.F. conducted the arbuscular mycorrhizal colonization assessments. P.T. and D.T. provided supervision and resources. L.M. and M.P. performed the HPLC–MS/MS analyses, contributed to resources, supervision, and manuscript writing. A.B. contributed to the study design, experiments, data collection and contributed to supervision. All authors reviewed and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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