6-benzylaminopurine and kinetin modulations during in vitro propagation of Quercus robur (L.): an assessment of anatomical, biochemical, and physiological profiling of shoots

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Research Article

Keywords: cytokinin, morphophysiological disturbances, oaks, plant anatomy, plant physiology, woody plant species

Posted Date: April 26th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1573274/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on June 2nd, 2022. See the published version at https://doi.org/10.1007/s11240-022-02339-9.

Abstract

For many woody species, such as *Quercus robur*, cytokinins in the culture medium are required to maintain in vitro plant material. Among synthetic cytokinins, 6-benzylaminopurine (BAP) and kinetin (KIN) are the most frequently used. In addition to inducing shoots, cytokinins can cause morphophysiological disorders. Therefore, we aimed to investigate the anatomical, biochemical, and physiological alterations and profiles of *Q. robur* shoots exposed to two cytokinins, applied alone and in combination. Shoots previously established in vitro were transferred to WPM culture media supplemented with BAP at concentrations of 0, 1.25, and 3.50 µM combined with KIN at concentrations of 0, 0.62, and 1.25 µM totaling 9 treatments. Anatomical, physiological, and biochemical analyses were performed after 40 d of culture. BAP induced the formation of new buds with anatomically underdeveloped leaves; induced shoot-tip necrosis, which is considered a response to the inefficient transport of water and nutrients; reduced the thickness of the cell walls of phloem fibers; and decreased the content of phenolic compounds and photosynthetic pigments. These responses were less pronounced with co-exposure to KIN. In contrast, KIN alone stimulated a larger area of secondary xylem and more lignified cell walls. BAP can induce shoots with underdeveloped anatomical and biochemical characteristics. Shoots that grew with KIN alone had stem and leaf anatomical characteristics, indicating greater commitment to cellular differentiation than proliferation. When both cytokinins are combined, KIN can partially mitigate the deleterious effects of BAP on in vitro growth.

Introduction

Quercus robur (L.) (Fagaceae) is an economically important hardwood tree species that is extensively used as construction material. However, the propagation of *Q. robur* by seeds may be challenging because of their short period of viability (Ntuli et al. 2011). An alternative for the propagation of *Q. robur* is via vegetative methods. This allows the maintenance of high-value genotypes and is useful for gene conservation (Savill and Kanowski, 1993). Among the vegetative methods, plant tissue culture techniques offer a valuable option for the large-scale propagation of economically interesting woody species, including *Quercus* (Pandey and Tamta 2012; Baccarin et al. 2015; Wojtania et al. 2015; Gentile et al. 2017; Fadladeen and Toma 2020).

During in vitro propagation, synthetic cytokinins are one of the main groups of plant growth regulators used to supplement the medium for induction of *de novo* shoot regeneration in *Quercus* shoots (Chalupa 1988; Puddephat et al. 1997; Martínez et al. 2017; Pandey et al. 2018). Although 6-benzylaminopurine (BAP) is the most common cytokinin used for the in vitro propagation of *Quercus*, there have also been reports of kinetin (KIN) usage (Chalupa 1988; Pandey and Tamta 2012; Fadladeen and Toma 2020). Pandey and Tamta (2012) observed that the previous use of KIN later influenced the rooting and subsequent survival of in vitro-propagated plants. However, studies on the effects of cytokinins on *de novo* shoot regeneration effectiveness, an approach to compare the two types, is frequently documented in woody species (Chalupa 1988; Sant'Ana et al. 2018; Fadladeen and Toma 2020). Nevertheless, the

combined use of two types of cytokinins sometimes yields better results than a single cytokinin (Sharma et al. 2017; Bhat et al. 2022).

Although cytokinins might be crucial for driving or improving in vitro responses, the shoots may show physiological and anatomical disorders induced by them (Dobránszki and Mendler-Drienyovszki 2014; Rosa et al., 2018). Under exogenous cytokinin exposure, plants may present with alterations in shoot length (visibly shorter shoots), decreased chlorophyll content, chlorotic leaves, shoot-tip necrosis, and low performance of the photosynthetic apparatus (Gentile et al. 2017; Lizárraga et al. 2017; Rosa et al. 2018; Silva et al. 2020; Murvanidze et al. 2022). Morphological disorders induced in vitro by synthetic cytokinins, such as shoot-tip necrosis, have been previously reported in woody plant species, including *Quercus* (Vieitez et al. 1985, 1997, 2009; Martínez et al. 2017; Surakshitha et al. 2019). However, according to Bairu et al. (2009), there are differing opinions on the effects of cytokinins on shoot-tip necrosis. This phenomenon is manifested by visible symptoms, in which the apical shoot becomes brown and later dies (McCown and Seller 1987).

Morphophysiological disorders might be a stress response and can be characterized by studies that connect the anatomy, physiology, and biochemistry of in vitro explants. Analyses involving the quantitative stem and leaf anatomy of shoots are valuable tools for defining how in vitro conditions can influence the success of all micropropagation steps (Martins et al. 2015; Manokari et al. 2021b, 2022). In addition to anatomical studies, a deeper approach to physiological and biochemical changes may be useful for stress characterization. Alterations in the antioxidant activity and content of photosynthetic pigments and phenolic compounds are considered good indicators of stress in plants under ex vitro and in vitro conditions (Wojtania et al. 2015; Chalker-Scott and Fuchigami 2018; Martins et al. 2018b, 2020).

Therefore, in addition to developing a micropropagation protocol, investigating the anatomical and physiological status of in vitro shoots may be crucial for further research. Many studies on the in vitro culture of woody plant species have focused on multiplication rates during micropropagation (Chalupa 1988; Sant'Ana et al. 2018; Bhat et al. 2022). In this way, many researchers have not considered the anatomical and physiological status of in vitro shoots at a deeper level. The morphophysiological status of in vitro-propagated shoots can interfere significantly during the acclimatization phase (Martins et al., 2018a; Manokari et al. 2021b).

In this study, we investigated the morphophysiological disorders that may occur during in vitro regeneration of *Q. robur* explants. Therefore, we proposed a comprehensive approach to assess the anatomical, biochemical, and physiological profiles of the in vitro-propagated shoots of *Q. robur*. We aimed to investigate the morphophysiological changes in *Q. robur* in vitro growing shoots exposed to two cytokinins (BAP and KIN), applied alone and in combination. The collected data were analyzed through additional data processing using multivariate analyses of Pearson correlation and principal component analysis (PCA).

Material And Methods

Plant material and in vitro establishment and culture

Branches measuring 1.5 m long were collected from selected *Q. robur* trees. The collected branches were cut into 30–40 cm fragments that were 2–3 cm in diameter and disinfected with sodium hypochlorite (commercial solution with 10% activated chlorine) for 20 min. The fragments of branches were cultured in plastic pots with water, under growth room conditions at 23 °C, in 90% humidity, and photoperiod of 16 h dark/8 h light. After 30 d, green epicormic shoots regenerated from the branches were used as explant sources to initiate the in vitro culture. The epicormic shoots were divided into approximately 2 cm fragments (with 1–2 buds each, without leaves) and disinfected with mercury chloride (0.1% for 3 min). After disinfection, the explants were washed four times with sterile distilled water for 1 min, placed on Woody Plant Medium (WPM) (Lloyd and McCown 1981) with 3.5 µM BAP (Sigma-Aldrich, St. Louis, USA) (Puddephat et al. 1997) and solidified with 7 g L^{-1} agar. The medium's pH was adjusted to 5.7 before autoclaving at 120 °C for 20 min. After transferring the explants to the in vitro culture medium, the plant material was kept under controlled conditions in a phytotron (growth chamber), with a light intensity of 80 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR), 16 h light/8 h dark photoperiod, at 23 °C. The explants were transferred to fresh culture medium every 40 d to obtain the necessary number of shoots with similar morphology.

In vitro culture with BAP and KIN

Shoots (approximately 2 cm) obtained under the aforementioned conditions were used as explants. Shoots were transferred to 320 mL glass containers containing 50 mL WPM with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar. The treatments consisted of medium supplemented with three levels of BAP (0, 1.25, and 3.5 µM) combined with three levels of KIN (0, 0.62, and 1.25 µM) (Sigma-Aldrich, St. Louis, USA), totaling 9 treatments. Shoots cultured in cytokinin-free medium (0 µM BAP + 0 µM KIN) were used as the controls. The experiment was conducted using four shoots per container. The media's pH was adjusted to 5.7 before autoclaving at 120 °C for 20 min. After shoot inoculation on the medium in a laminar flow cabinet, the plant material was kept in a growth chamber for 40 d under the conditions mentioned above.

Analysis of the multiplication rate

The multiplication rate was estimated by verifying the budding percentage response and number of shoots per explant. The analysis was performed after 40 d of growth using 40 explants from each treatment, which were randomly collected and divided into five parcels (n = 5). In addition, the probability of shoot-tip necrosis was analyzed. Each jar in which at least one shoot-tip exhibited necrosis was classified as under the presence of necrosis. Probability was calculated using 10 different jars (n = 10).

Stem and leaf anatomy of in vitro *Q. robur* shoots

After 40 d of culture, five shoots from each treatment were sampled randomly and stored in 50% ethanol. The anatomical characterization of stems was performed by examining cross-sections of stems between 0.5 and 0.8 cm from the shoot base. The leaf anatomy was determined by cross sections of the first fully expanded pair of leaves from the apex. Cross-sections were prepared with the aid of a double razor and safranin–astra blue staining solution. The leaf venation pattern (vein distribution) was characterized by diaphanization in 5% sodium hydroxide solution (NaOH) and staining with 1% safranin solution (Kraus and Arduin 1997; Martins et al. 2020). The slides were photographed using a digital camera (Zeiss AxioCam MRc 5, Germany) coupled to a Zeiss Axioskop microscope (Carl Zeiss, Germany). Anatomical measurements (n = 5) were carried out using Imagetool® software calibrated with a microscopic ruler. In the stem cross sections, the total transverse stem (μm^2) and xylem area (μm^2) were measured. The anatomical traits analyzed in the leaves were the thickness of the adaxial (μm) and abaxial epidermis (μm), palisade (μm), spongy parenchyma (μm), cell walls of the phloem fibers (μm), and the number of vessel elements.

The leaf and stem cross sections were stained with berberine hemisulfate and aniline blue solutions. This was done to visualize lignin distribution in the tissues (Brundrett et al. 1988). Staining procedures and slide assembly were performed as described by Martins et al. (2020). Sections were observed under a fluorescence microscope (Zeiss Axioskop, Germany). Images were captured with a Zeiss AxioCam MRc 5 camera using UV light with an excitation/emission spectrum of 385 nm.

Quantification of photosynthetic pigment contents

Pigment extraction was performed using 0.030–0.039 g of six independent plant samples (n = 6). The material was placed in 2 mL Eppendorf containing 1 mL 80% (v/v) acetone and maintained for 48 h in the dark at 4 °C. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) contents were quantified using a microplate reader (Infinite M200 pro, Tecan, Switzerland). The absorbance was measured at λ = 665, 645, and 480 nm for Chl *a*, Chl *b*, and Car, respectively. The Chl content was estimated as proposed by Arnon (1949) and expressed as micrograms of pigment per gram of fresh weight (μg g^{-1} FW).

Extraction of phenolic compounds

Leaf samples containing 0.1 g material were homogenized in methanol:water:hydrogen chloride (79:20:1) solvent at 4 °C. Hydrogen chloride was added to prevent oxidation. Clear extracts were obtained after 15 min of centrifugation at 16,000 × *g* and were further diluted for quantification. Total phenolic content (TPC) was determined spectrophotometrically (A_1) using the Folin-Ciocalteu (F-C) reagent (Singleton et al. 1999). A mixture containing 20 μL diluted sample extract, 40 μL 10% FC reagent, and 160 μL 700 mM sodium carbonate was incubated for 1 h in the dark and measured at 765 nm using an Infinite M200 PRO

(Tecan) plate reader and Magellan software. One milliliter of diluted extract was incubated with 0.05 g hide powder (60 min) to adsorb tannins and centrifuged for 5 min at 16,000 × *g* (Ainsworth and Gillespie 2007). The non-tannin phenolic content was determined spectrophotometrically (A_2) using the procedure described above. Serial dilutions of 0–6 mM gallic acid in methanol was used to create a standard curve (R^2 = 0.9998) expressing the phenolic compounds as gallic acid equivalents (GAE).

Antioxidant activity of phenolic compounds

Determination of the antioxidant activity in the total phenolic and non-tannin phenolic extracts was carried out using a CUPric reducing antioxidant capacity (CUPRAC) assay, in which the main reagent, copper (II)-neocuproine (2,9-dimethyl1,10-phenanthroline), oxidizes antioxidants to generate a colored product (Apak et al. 2004). The reaction was performed in 1 M ammonium acetate buffer (pH 7) containing 10 mM copper chloride and 7.5 mM neocuproine. Absorbance was measured at 450 nm and calculated based on the calibration curve obtained with Trolox in the 0–25 µM range (R^2 = 0.9952) and expressed as Trolox equivalent antioxidant activity (TEAC).

Chlorophyll *a* fluorescence analysis

Photosynthetic apparatus performance was measured using an FMS 2+ pulse-modulated chlorophyll fluorimeter (Hansatech, King's Lynn, Norfolk, UK) according to the experimental protocol described by Kramer et al. (2004). Measurements were performed on six plants from each treatment at 40 d of growth. Dark adaptation and reading procedures were performed as described by Martins et al. (2020). The maximum quantum yield of primary photochemistry [Fv/Fm = 1 – (Fo – Fm)], quantum yield for energy dissipation [Fo/Fm = 1 – (Fv – Fm)], and non-photochemical quenching coefficient [q_{NP} = (Fm – Fm)/(Fm – Fo)] were calculated automatically by the device.

Statistical analysis

The experimental design was completely randomized using a 3 × 3 factorial scheme (BAP concentrations of 0, 1.25, and 3.5 µM × KIN concentrations of 0, 0.62, and 1.25 µM). The data were subjected to two-way analysis of variance (ANOVA), and the means were compared using Tukey’s test at 5% significance. For additional analyses, a correlation matrix and PCA were performed. Correlation matrices were created using Pearson's method in the corrplot package. The function PCA was implemented with "FactoMineR" version 2.3, factoextra version 1.0.7, corrplot version 0.84 R packages (Le et al. 2008; Wei and Simko 2017; Kassambara and Mundt 2020).

Results

Growth traits

Cytokinins considerably influenced the in vitro regeneration process (Fig. 1). More precisely, explants grown in cytokinin-free medium (0 µM BAP + 0 µM KIN) exhibited leaves with necrotic lesions (Fig. 1A). Shoots cultured under BAP-free conditions did not form any shoot buds at the base of the explants (*de novo* buds), regardless of KIN concentration. In contrast, BAP supplementation had a positive effect on the budding response and number of new shoots. Among the treatments with 3.5 µM BAP, explants grown with 1.25 µM KIN displayed a 29% reduction in budding response (Figs. 1J-K). Shoot-tip necrosis was visible in explants exposed to BAP. This phenomenon was enhanced by increasing the BAP concentration, irrespective of the KIN concentration applied (Fig. 1L).

Influence of cytokinins on stem and leaf anatomy

Although transversal stem cross-sections of *Q. robur* shoots were performed in the same region of the organ, apparent differences in stem morphology were observed among the treatments. Stem width (total transverse stem area) was affected only by BAP, and it increased linearly (R^2 = 0.98) as a function of BAP concentration (Figs. 2A-J). Importantly, KIN concentration was the primary modulator of the xylem area. Shoots grown under 1.25 µM KIN had the largest xylem area in the cross-sections of stems (Figs. 2A-I, L). Both BAP and KIN affected the xylem area/total transverse stem area ratios. However, the effects of both cytokinins were independent of each other. A linear reduction in the xylem area/total transverse stem area ratio was strongly related to increasing BAP concentrations (R^2 = 0.95). Among the KIN concentrations, shoots cultivated with 1.25 µM KIN achieved the highest xylem area/total transverse stem area ratio (Figs. 2A-I, K).

The leaves showed morphological differences. Although we did not measure leaf size, the leaves grown with KIN alone were clearly larger. In contrast, shoots cultured without exogenous cytokinin supplementation or BAP treatment had smaller leaves. Venation patterns also changed. Shoots grown in BAP-free medium showed leaves with thicker veins (Fig. 3).

Under the tested conditions, *Q. robur* leaves exhibited stomata with a hipostomatic distribution wherein the epidermis had one cell layer on both the adaxial and abaxial sides. The mesophyll revealed dorsiventral organization, with one layer of palisade parenchyma on the adaxial leaf side and three layers of spongy parenchyma on the abaxial side. The vascular bundles were collateral and surrounded by an almost uninterrupted ring of phloem fibers (Fig. 4).

The treatments had an impact on the leaf anatomy. The thickness of both the adaxial and abaxial sides of the epidermis did not change and reached 11.3 µm and 10.4 µm on average, respectively (Fig. 4). The thickness of the palisade parenchyma was affected by BAP and KIN concentrations; however, they acted separately. Shoots exposed to KIN had thicker palisade parenchyma, whereas leaves with thinner palisade parenchyma were observed after BAP supplementation (Figs. 4, 5A). The thickness of the spongy parenchyma was modified using both the BAP and KIN. Leaves grown without BAP showed similar spongy parenchyma thickness among treatments with KIN. The leaves of shoots grown with 1.25

μM BAP had thicker spongy parenchyma when co-exposed to 1.25 μM KIN. In contrast, a reduction in the thickness of this parenchyma was observed in shoots cultured with 3.5 μM BAP + 1.25 μM KIN (Figs 4, 5F). Both BAP and KIN concentrations influenced the palisade/spongy ratio, but acted independently. The palisade/spongy ratio decreased as the BAP concentration increased (R^2 = 0.86), whereas KIN exposure increased the palisade/spongy ratio (Figs. 4, 5B).

However, concerning the anatomical traits of leaves, the number of vessel elements and the thickness of the cell walls of phloem fibers were assumed to be influenced by BAP and KIN. The highest number of vessel elements was observed in leaves grown in a medium containing 1.25 μM KIN and without BAP (Figs 4 and 5G). Furthermore, leaves that grew under BAP exposure displayed considerably decreased cell wall thickness of phloem fibers. By comparing 0 or 1.25 μM BAP treatments, leaves grown under 0.62 μM KIN showed greater thickness for the cell walls of phloem fibers (Figs 4, 5C).

The deposition of lignin in the cross-sections of stems and leaves was visualized using berberine hemisulfate and aniline blue solutions, which emitted fluorescence from the cell walls of xylem and phloem fibers. The area of fluorescence signal was reduced in plants treated with BAP. Interestingly, the fluorescence signal in the leaves was lower (Fig. 6).

Photosynthetic pigment contents

BAP and KIN affected the content of photosynthetic pigments in *Q. robur* shoots cultured in vitro. More precisely, BAP and KIN concentrations affected all parameters related to Chl *a* and Chl *b*. Among the treatments without KIN, shoots cultured with 3.5 μM BAP showed a significant reduction in Chl *a*, Chl *b*, Chl *total* content, and Chl *a/b* ratio. A decrease in Chl content and the Chl *a/b* ratio was also detected in shoots grown in media with 0.62 or 1.25 μM KIN BAP (1.25 and 3.5 μM) (Figs. 5D, E, I, J).

Likewise, the Chl *a* and Chl *b* contents of carotenoids were also modulated by the BAP and KIN concentrations; however, they acted independently. The Car content increased (R^2 = 0.85) concomitantly with the KIN concentrations, but decreased (R^2 = 0.97) with increasing BAP concentration (Fig. 5H).

Phenolic content and Trolox equivalent antioxidant capacity

Phenolic content and antioxidant capacity were investigated in leaves modified with BAP and KIN. Application of 0 and 0.62 μM KIN reduced the content of total phenolics and non-tannin phenolics when shoots were co-exposed to BAP. Among the treatments with 1.25 μM KIN, the contents of the total phenolics and non-tannins were similar but lower than those in the control plants grown without cytokinin supplementation. The highest total antioxidant capacity, expressed as the Trolox equivalent antioxidant capacity (TEAC), was observed in the leaves of shoots cultured with 3.5 μM BAP and without KIN. After the elimination of condensed tannins, BAP alone was found to stimulate the antioxidant response, together with an increase in its concentration (Figs 7A-D).

Chlorophyll *a* fluorescence

Cytokinin treatments have been documented to modulate chlorophyll *a* fluorescence. Fv/Fm and Fo/Fm were affected only by BAP concentrations. Shoots grown in the medium supplemented with BAP had higher Fv/Fm values. Nevertheless, a tendency of decrease at BAP concentrations higher than 1.25 µM was revealed (Fig. 8A). The Fo/Fm value was higher when BAP was not added to the medium (Fig. 8B). q_{NP} was impacted by both BAP and KIN. The lowest q_{NP} was observed in plants cultured in the control KIN-free and BAP-free media. In contrast, the highest q_{NP} was detected in the leaves of shoots grown on the medium supplemented with only 1.25 µM BAP (Fig. 8C).

Correlation and principal component analysis (PCA)

Correlation analysis between all measured parameters was performed. Growth traits related to *de novo* shoot regeneration induction (budding and number of new shoots) showed a negative relationship with anatomical traits (palisade parenchyma, palisade/spongy ratio, and thickness of cell walls of phloem fibers) and phenolic content (total phenolics and non-tannin phenolics). In addition, the non-tannin TEAC showed a negative relationship with Chl *a/b.* The leaf anatomical traits were positively correlated with the phenolic content (total phenolics and non-tannin phenolics) (Fig. 9A).

PCA was performed using the parameters that most contributed to the explanation of the data; thus, spongy parenchyma, adaxial and abaxial epidermis, stem width, and q_{NP} were excluded from the analysis. The two principal components explained 55% of the total variance in the dataset, with axes 1 and 2 explaining 40.4% and 14.6% of the variance, respectively. The traits related to growth (budding, number of new shoots, and shoot-tip necrosis (STN)), anatomy (anatomical traits of stems and leaves), antioxidant capacity (TEAC and non-tannin phenolics TEAC), and content of photosynthetic pigments (Chl *a*, Chl *b*, Ch *a/b*, Chl *total*, and Car) were correlated with axis 1 of the analysis (average > 0.7). The photochemical parameters (Fv/Fm and Fo/Fm) obtained through Chl *a* fluorescence showed a high correlation with axis 2 (average > 0.6). Through PCA analysis, three distinct groups were revealed, and they showed correlations of the analyzed traits in this study with the treatments. The first cluster showed the treatments without BAP supplementation (0 µM BAP + 0 µM KIN, 0 µM BAP + 0.62 µM KIN, and 0 µM BAP + 1.25 µM KIN), which were plotted as a function of axis 1, on the right side. This demonstrates a correlation with anatomical traits, phenolic content, and photosynthetic pigment content. The second cluster grouped the treatments with 1.25 µM BAP supplementation (1.25 µM BAP + 0 µM KIN, 1.25 µM BAP + 0.62 µM KIN, and 1.25 µM BAP + 1.25 µM KIN) and this was plotted as a function of axis 2. The second cluster demonstrated its correlation with Chl *a* fluorescence parameters. The treatments with 3.5 µM BAP supplementation were grouped in the third cluster (3.5 µM BAP + 0 µM KIN, 3.5 µM BAP + 0.62 µM KIN, and 3.5 µM BAP + 1.25 µM KIN), and were also plotted in axis 1, but on the left side. PCA analysis showed a high correlation among *de novo* shoot regeneration capacity, shoot-tip necrosis, and antioxidant capacity (Fig. 9B).

Discussion

This study reports the impact of BAP and KIN alone and their synergistic effect on the in vitro regeneration process as well as on the morphophysiology of *Q. robur* shoots. Shoots cultured with BAP showed anatomical and biochemical characteristics that denote a less advanced level of secondary growth (transition status between primary and secondary growth). In contrast, shoots cultured in medium without BAP (control and KIN supplementation) had tissues with highly differentiated cells and clear secondary growth morphology. These characteristics were correlated with *de novo* shoot regeneration efficiency.

BAP was more efficient in promoting shoot bud formation at the base of the explants. The effective response of BAP to in vitro multiplication of *Quercus* species has been reported previously (Puddephat et al. 1997; Tamta et al. 2008; Pandey and Tamta 2012). BAP shows a more prolonged stimulation of cell division because it is not easily broken down by plants and is more stable and resistant to oxidation (Agustina et al. 2020). Such characteristics may contribute to the effectiveness of this synthetic cytokinin in stimulating bud formation, as observed in our study. The efficiency of BAP in our study correlated with a lower level of cell determination in the tissues. During in vitro culture, the composition of the medium can modulate cells to stages with variable differentiation levels, including multipotent, pluripotent, and totipotent levels (Baccarin et al., 2015). This response can regulate plant tissues with lower cellular determination and higher competence acquisition. In addition, shoots of *Q. robur* cultured with BAP showed stems with a larger investment in parenchymatic cells, that is induction of adventitious buds. Parenchyma cells in shoot bases may act as a pluripotent cell niche for direct organogenesis (induction of adventitious buds) (Graner et al. 2019).

The explants of *Q. robur* were unable to induce shoot buds in the control medium (cytokinin-free) or under KIN supplementation as the only exogenous cytokinin source. This species is not only exogenous cytokinin-dependent for shoot organogenesis induction, but also synthetic cytokinin type-dependent for the effectiveness of the regeneration process. Similar results have been previously reported for *Q. robur* and *Q. aegilops* (Chalupa 1988; Fadladeen and Toma 2020). In the present study, the low efficiency of *de novo* shoot regeneration in *Q. robur* was predominantly linked to anatomical traits. Shoots cultured in a medium without BAP were characterized by tissues of the stems with cells more committed to differentiation (high cellular determination and low competence acquisition) than proliferation, which leads to a less efficient morphogenetic status for the induction of shoots at the base of the stem. Cell proliferation is required for *de novo* shoot regeneration (Fehér, 2019). In this context, BAP favored cell proliferation in *Q. robur* explants. In addition, it has been suggested that the low efficiency of KIN in shoot induction could be correlated to the activity of the enzyme system that catalyzes the degradation of cytokinins, such as cytokinin oxidase/dehydrogenase (Schmülling et al. 2003; Nisler et al. 2016; Rosa et al. 2018).

With respect to the anatomical traits of stems, shoots grown with 1.25 µM KIN, mainly in the absence of BAP, presented a larger area of secondary xylem and lignified cells. In contrast, BAP induced shoots with

a reduced xylem area/total transverse stem area ratio in *Q. robur* explants. More developed xylem can transport water and mineral nutrients better to the whole aerial part, in addition to providing mechanical support (Melnyk 2017; Cabello and Chan 2019; Qi et al. 2020). However, the low mobility of nutrients may lead to physiological disorders in vitro, such as shoot-tip necrosis (Silva et al. 2020). These authors also verified that the incidence of shoot-tip necrosis was higher in shoots with non-lignified tissues (softer tissues) than in those with more lignified tissues. These findings are in agreement with those of the present study. The probability of shoot-tip necrosis showed a negative relationship with the xylem area/total transverse stem area ratio in *Q. robur* explants. The xylem area fraction is related to the hydraulic efficiency of stems (Cabello and Chan 2019; Zhang et al. 2021). This is because xylem hydraulic conductivity is associated with the number and diameter of vessel elements (based on the Hagen–Poiseuille equation), and even a slight reduction in these xylem features can affect the transport of water and nutrients (Quintana-Pulido et al. 2018; Martins et al. 2019; 2021). Thus, we suggest that shoot-tip necrosis is a morphophysiological response due to inefficient transport of water and nutrients through the shoot from the base to the tip.

Likewise, the anatomical traits of stems and leaves also exhibited characteristics that indicated a lower level of tissue development. Manokari et al. (2021a, b) demonstrated that leaves grown under BAP showed underdeveloped structural traits, such as reduced/underdeveloped xylem and phloem elements and tissues of support (collenchyma and sclerenchyma). In the present study, the epidermal cells were similar in thickness; however, the cells of the chlorophyll parenchyma were affected. Cytokinin type affected the elongation of palisade parenchyma cells. Leaves grown with BAP had palisade parenchyma cells that were more compact. In contrast, KIN induced longer cells. This decline in the palisade parenchyma thickness may be correlated to a lower efficiency of water transport promoted by the xylem vessels, which can influence the cell elongation process. Cell elongation is promoted by cell expansion and directly interferes with water uptake (Schmalstig and Geiger 1985; Silva-Cunha et al. 2021).

However, the spongy parenchyma thickness did not show a clear response to the function of the treatments. Nevertheless, when the palisade/spongy bone ratio was analyzed, a clear pattern of anatomical response was observed. Addition of BAP to the medium induced a lower palisade/spongy ratio, mainly in the absence of KIN. This decreased palisade/spongy ratio can indicate a reduced development level of leaf anatomy, which means a larger area with larger interstitial spaces among parenchyma cells (Luan et al. 2021; Manokari et al. 2022).

The effects of cytokinin treatments on the formation of leaf conduction tissues were also documented. The number of vessel elements in the midrib differed among the treatments with and without KIN, which means that KIN can stimulate the development of leaves with higher hydraulic conductivity, as discussed before. However, a closer observation of the leaf venation pattern, including the vein density and caliber of the vessels, raised the hypothesis that BAP modulates the formation of conduction tissues. More precisely, BAP limited the development of leaf veins, mainly in leaves grown under 3.5 µM. The declining capacity of hydraulic conductivity in plants reflects leaf xylem formation (Falchi et al. 2020). The

quantification of the number of vessel elements and/or vein density can indicate the efficiency of water and nutrient transport (Martins et al. 2021; Sakurai and Miklavcic 2021).

Our study revealed that BAP negatively influenced the thickness of the cell walls of the phloem fibers (sclerenchyma). This response was linked to the total phenolic content, as confirmed by PCA and correlation analysis. Phenolic content was significantly positively correlated with lignin production, one of the cell wall components of all vascular plants (Zhao et al. 2016; Chalker-Scott and Fuchigami 2018; Tikhomirova et al. 2018). In leaves, the proper development of sclerenchyma cell walls is essential for maintaining shape and functioning (Martins et al. 2015). As part of vascular bundles, sclerenchyma cell walls can support the tissues responsible for transporting water and nutrients throughout the plant (Bao et al. 2012). Thus, leaves with lower cell wall thickness, in response to low lignin deposition, can impair conduction tissues. Therefore, the anatomical traits (stem and leaf) of shoots grown with BAP, especially at 3.5 µM, can indicate low-efficiency hydraulic conductivity in the whole plant body, which could correlate with shoot-tip necrosis.

The total phenolic and non-tannin phenolic contents were negatively correlated with budding percentage and number of new shoots. Wojtania et al. (2015) suggested that phenolic content may inhibit shoot formation in vitro. These authors also revealed that the addition of BAP lowered phenolic production compared with the BAP-absence medium. This corroborates our findings based on PCA analysis. However, we also showed that budding induction is related to anatomical traits.

Our work also found changes in Chl and Car contents. Decreases in the content of photosynthetic pigments in leaves cultured in vitro supplemented with excess cytokinin are frequently reported (Gentile et al. 2017; Martins et al. 2018b; Murvanidze et al. 2022). This decline in the content of photosynthetic pigments may be related to physiological stress, which results in alterations in reactive oxygen species production and increased antioxidant activity (Dakah et al. 2014; Gentile et al. 2017). *Q. robur* shoots cultivated with excess cytokinin showed clear signs of physiological disorders observed, such as s reduced Chl *a/b* ratio and increased antioxidant activity (TEAC), in addition to lesser content of photosynthetic pigments. A reduction in the Chl *total* content, followed by a reduction in the Chl *a/b* ratio, indicates high Chl *a* degradation and possible damage to the density of reaction centers of photosystem II (PSII) (Martins et al. 2021). We hypothesize that excess cytokinin can induce oxidative stress, leading to changes in Chl content. Our hypothesis is supported by the negative relationship between the content of photosynthetic pigments and the Chl *a/b* ratio and measured antioxidant capacity.

Shoots that grew with 3.5 µM BAP combined with 0.62 or 1.25 µM KIN did not present an antioxidant activity as high as the shoots cultured with 3.5 µM BAP alone (KIN free). This could be interpreted as an indirect response to the Car content. Carotenoids can act as antioxidants by scavenging reactive oxygen species (Kim et al. 2021). Martins et al. (2021) also verified that a reduced Car content could negatively affect the Chl content as well as the Chl *a/b* ratio. Therefore, even though 3.5 µM BAP induced shoots with a lower Car content, this response was less pronounced with KIN co-exposure. This could have

contributed to the smaller alterations in the Chl proportion, which was negatively correlated with antioxidant activity.

Cytokinin treatment also influenced the photosynthetic apparatus performance. The addition of BAP promoted slight changes (approximately 5%) in the parameters related to potential quantum yield (Fv/Fm and Fo/Fm). Leaves under typical conditions usually present range values of Fv/Fm between 0.7–0.8 and Fo/Fm between 0.2–0.3. However, an Fv/Fm below 0.6 indicates photoinhibition as a stress response (Guidi et al. 2019). Thus, shoots under in vitro conditions did not show strong signs of photoinhibition based on the quantum yield of PSII. Nevertheless, the results suggest that *Q. robur* shoots must grow under ideal exogenous cytokinin supplementation because either too low or too high concentrations reduces the potential photochemical efficiency of PSII, and more energy is lost through dissipation.

Further analyses showed the importance of exogenous cytokinin supplementation in the photosynthetic apparatus performance of *Q. robur* shoots in vitro. Shoots cultured with BAP and/or KIN had q_{NP} values ≥ 0.58. These values indicate that the plants have a high photoprotective capacity. The q_{NP} parameter can vary between 0 and 1. It is related to the capacity of a plant to protect itself from photodamage due to excess energy (photoinhibition) (Lurie et al. 1994; Kumar and Parsad 2015; Maoka 2020). In the present study, the drastic reduction in q_{NP} in the shoots of the control treatment (cytokinin-free) can denote the lowest efficiency of plants to minimize any photoenergy-induced damage to the reaction centers. This low photoprotective capacity could be correlated with the leaf senescence process, since shoots of the control treatment showed leaves with necrotic lesions. Although plant hormones interact with each other to regulate leaf senescence, an increase in endogenous cytokinin levels can delay the processes involved in leaf senescence (Schippers et al., 2007; Xiao et al. 2017).

Through correlation and PCA analyses, it was possible to prove that the ability to induce *de novo* shoot regeneration is negatively correlated with the development of plant tissues at the cellular and biochemical levels. The use of BAP in the culture medium generated shoots with organs that clearly showed underdeveloped anatomical and biochemical characteristics. The preservation of cells at a lower level of determination is essential to allow greater cell proliferation and the formation of new shoots. Therefore, the results suggest that the key to competence acquisition and induction of new shoots might involve preserving cells to stages with multipotent and/or pluripotent characteristics. However, the formation of tissues with less specialized cells also impairs shoot quality due to the malformation of conduction tissues, which can lead to shoot-tip necrosis. In contrast, in the in vitro culture without BAP, the formation of tissues showed a higher cellular determination stage (highly differentiated and specialized cells), which led to a lower *de novo* shoot regeneration capacity.

Conclusion

BAP can induce shoots with underdeveloped anatomical and biochemical characteristics. A lower level of cellular determination in tissues was linked to *de novo* shoot regeneration efficiency. However, the BAP

shoots also presented clear signs of physiological stress, such as low photosynthetic pigment content and higher antioxidant activity. An increase in BAP concentration can enhance the shoot-tip necrosis phenomenon. In contrast, shoots grown with KIN alone had stem and leaf anatomical characteristics that indicated a greater commitment to cellular differentiation than proliferation and showed a clear secondary growth morphology. When both cytokinins are combined (KIN and BAP), KIN can partially mitigate the deleterious effects of BAP on in vitro growth.

Declarations

Acknowledgments:

The authors acknowledge the Ulam Programme scholarship (PPN/ULM/2019/1/00037) granted by Polish National Agency for Academic exchange. The authors also acknowledge Andreia Barcelos Passos Lima Gontijo, Lorenzo Toscano Conde, and Ludmila Cristina Oliveira for providing chemical reagents related to plant anatomy. Additional support was provided by the Institute of Dendrology - Polish Academy of Sciences.

Author contributions:

JPRM, MKW, and JMLL performed experiments and analyzes. JPRM wrote the manuscript and performed the statistical analysis. EMK quantified phenolic compounds and their antioxidant activity. MMM performed the PCA analysis. MKW and PC provided the structure and contributed to the design and interpretation of the results.

Conflict of interest:

All authors certify that they have no conflict of interest.

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Figures

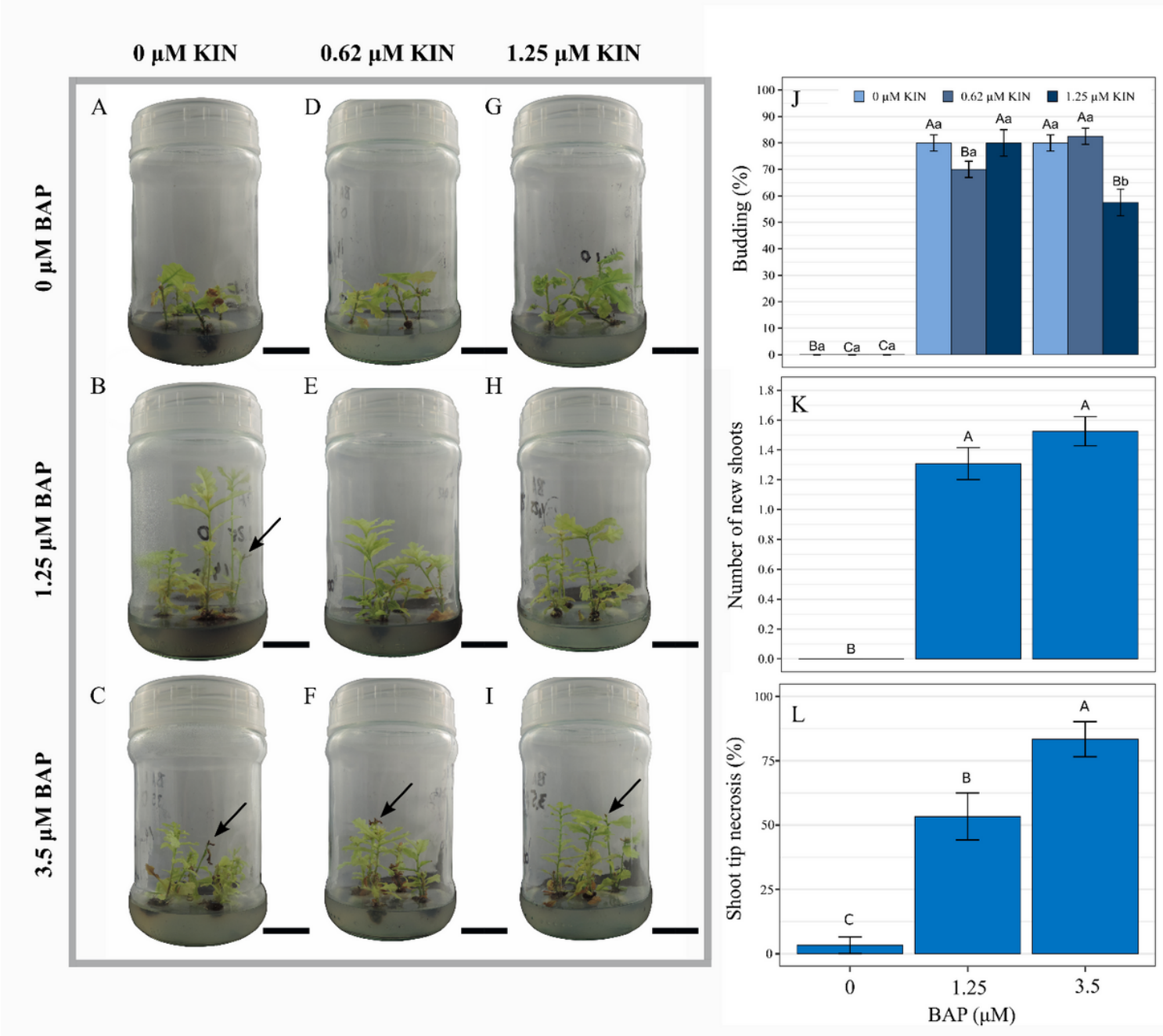

Figure 1

The visual aspect of *Q. robur* in vitro shoot growth after 40 d as a function of cytokinins (BAP, KIN) treatments. (A-I). The arrows detailed morphology of shoot-tip necrosis formed in vitro. Budding (%) (J), number of new shoots (K), and probability of shoot tip necrosis (L) of *Q. robur* during in vitro growth. For each growth trait, the means ± SE followed by the same letter (uppercase letters comparing BAP concentrations and lowercase letters comparing KIN concentrations at each BAP concentration) do not differ significantly according to Tukey's test ($p<0.05$). Bar = 2 cm

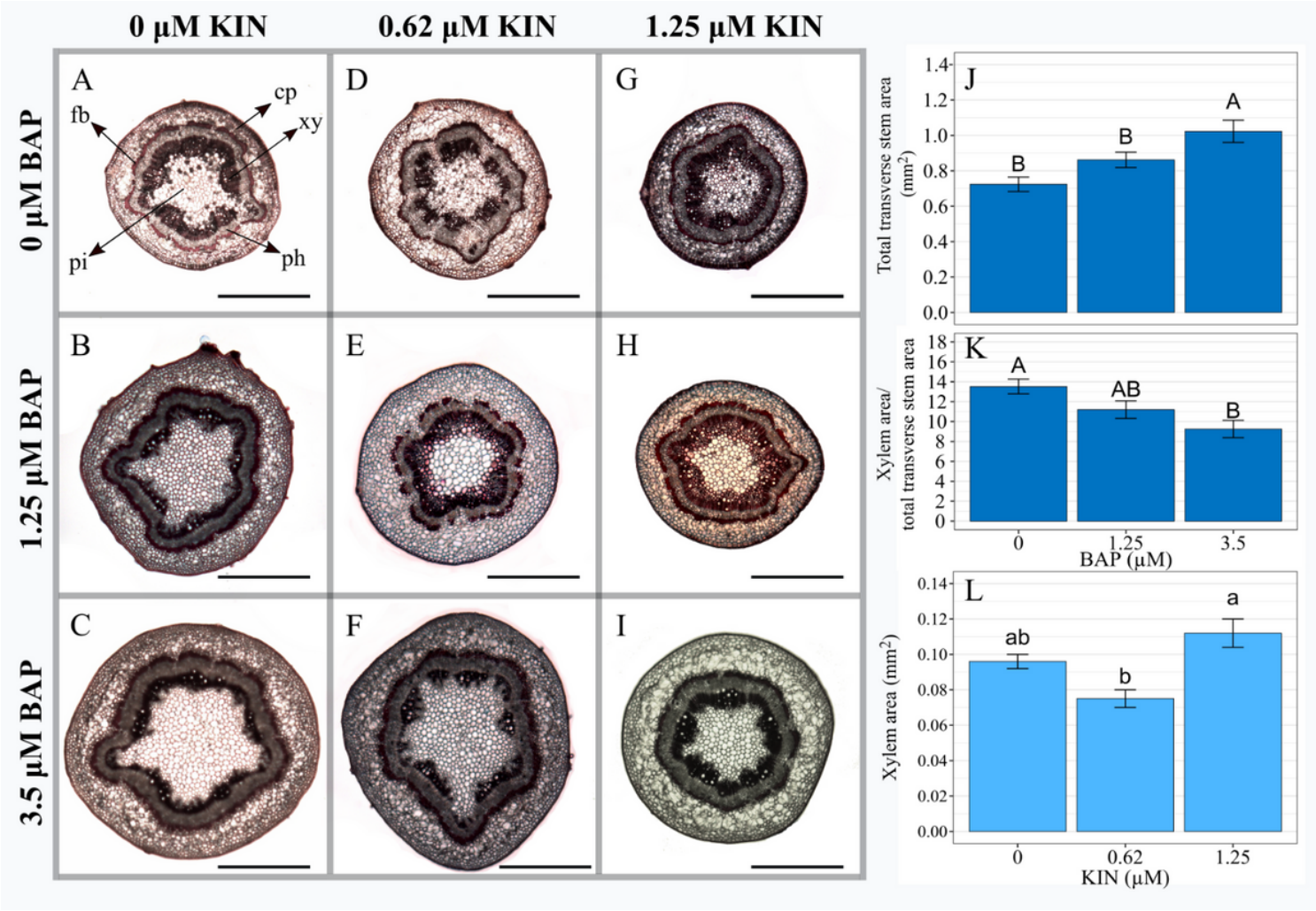

Figure 2

Cross-sections of stems of *Q. robur* after 40 d in medium containing BAP (0, 1.25, and 3.5 µM) and KIN (0, 0.65, and 1.25 µM) during in vitro culture. Stem cross-sections stained by safranin and astra-blue solutions. For each anatomical trait, the means ± SE (*n* = 5) followed by the same letter do not differ significantly according to Tukey's test ($p<0.05$). cp – cortical parenchyma, fb – fibers, pi – pith, ph – phloem, xy – xylem. Bars = 500 µm

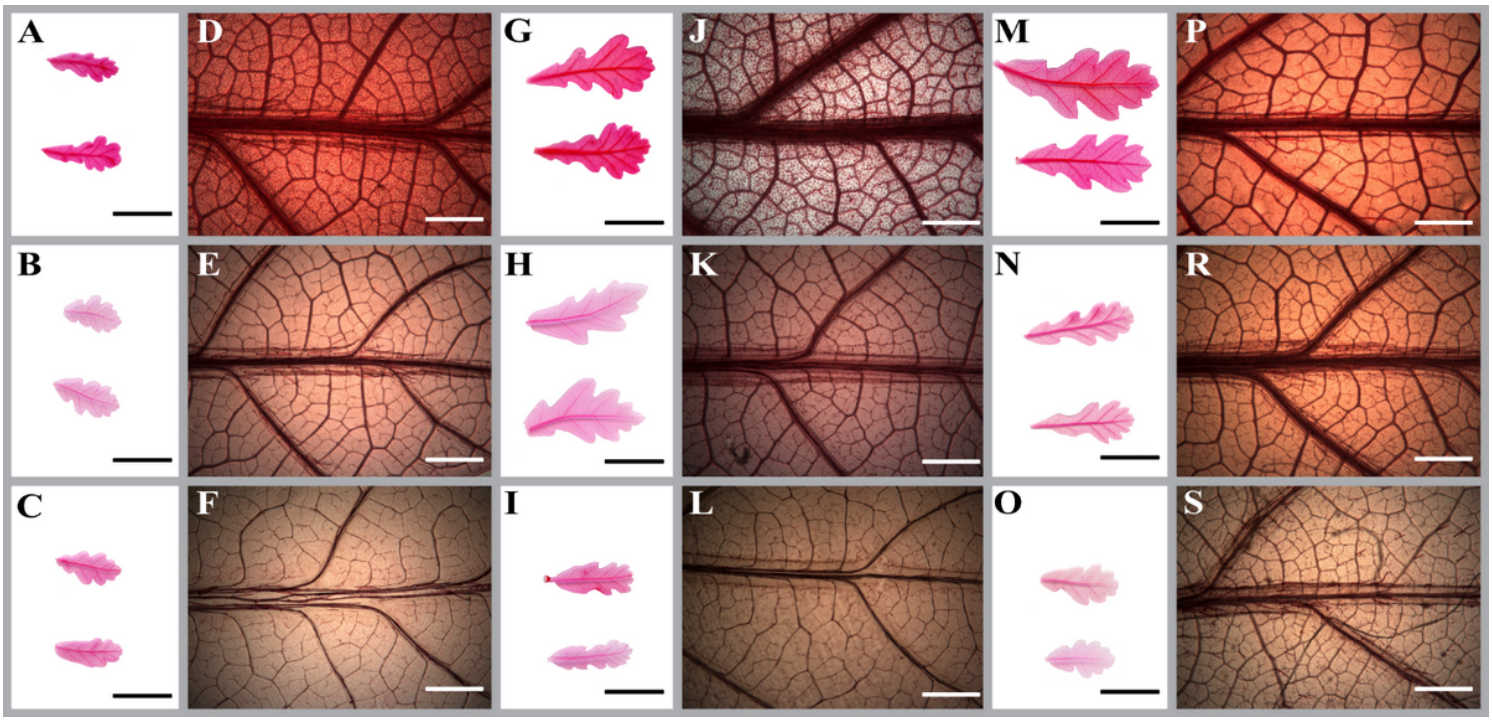

Figure 3

Venation pattern of leaves of *Q. robur* shoots grown in medium containing different combinations of BAP (0, 1.25, and 3.5 μM) and KIN (0, 0.65, and 1.25 μM) during in vitro culture. Bars = 1 cm (A-C, G-I, and M-O) and 500 μm (D-F, J-L, and P-S).

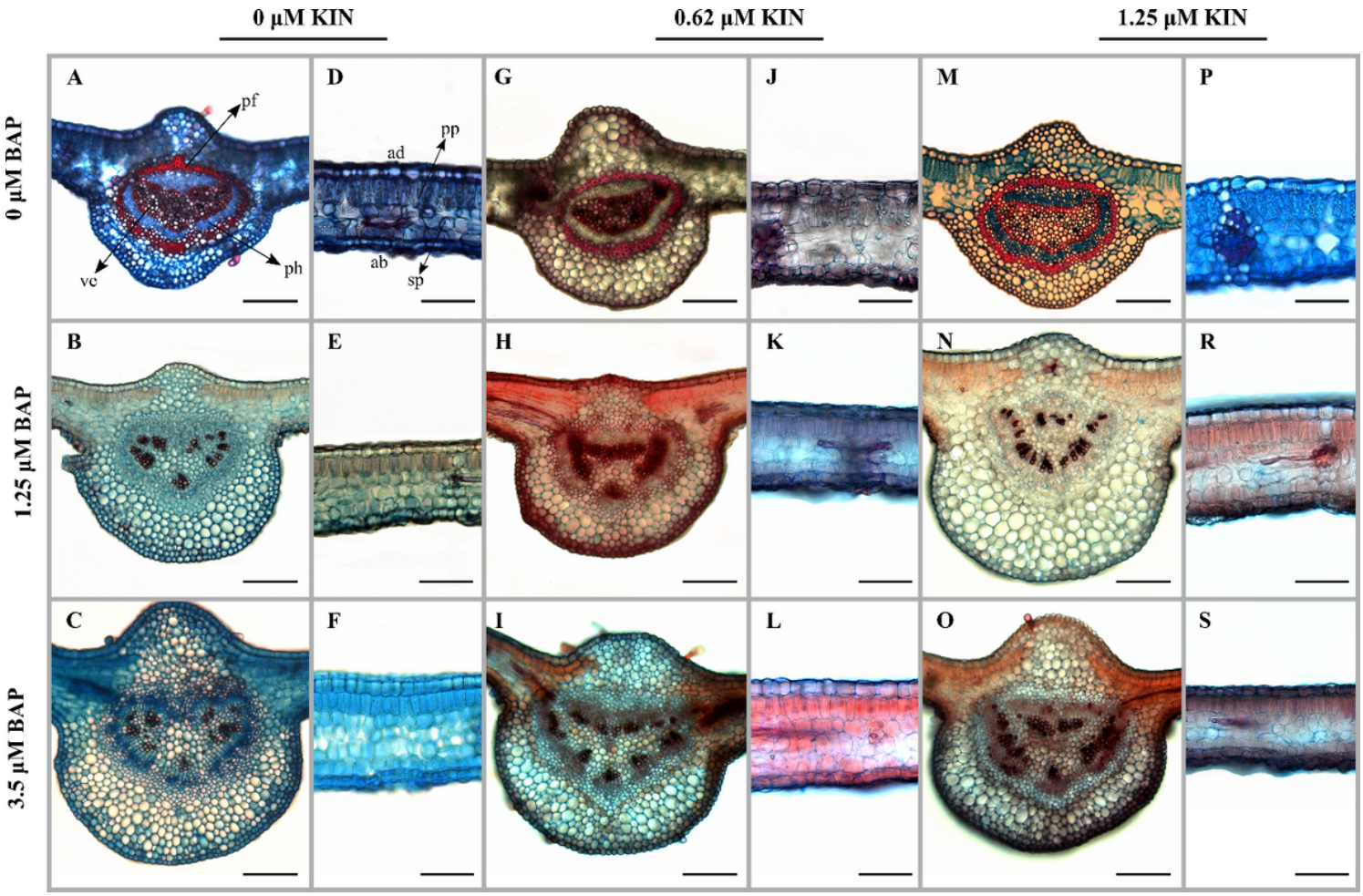

Figure 4

Cross-sections of *Q. robur* leaves at 40 d in medium containing different combinations of BAP (0, 1.25, and 3.5 µM) and KIN (0, 0.65, and 1.25 µM) during in vitro culture. Stem cross-sections stained by safranin and astra-blue solutions. ab – abaxial epidermis, ad – adaxial epidermis, pp – palisade parenchyma, ph – phloem, pf – phloem fibers, sp – spongy parenchyma, ve – vessel element. Bars = 100 µm (A-C, G-I, and M-O) and 50 µm (D-F, J-L, and P-S).

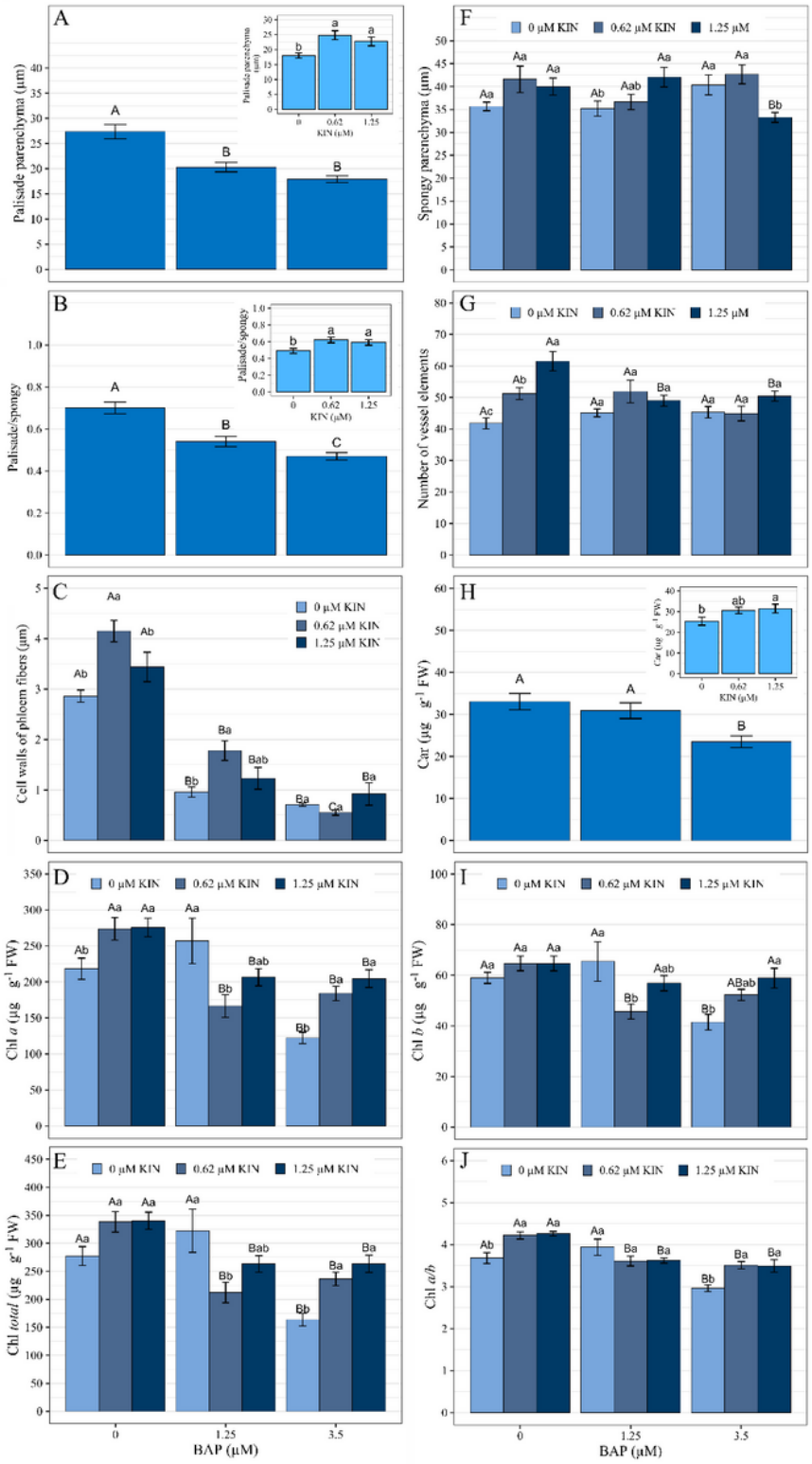

Figure 5

Anatomical traits and photosynthetic pigment contents of *Q. robur* leaves as a function of cytokinins (BAP, KIN) during *in vitro* culture. For each growth trait, the means (± SE), $n = 5$, or for each pigment content, means (± SE), $n = 6$, followed by the same letter (uppercase letters comparing BAP concentrations at each KIN concentration and lowercase letters comparing KIN concentrations at each BAP concentration), do not differ significantly according to Tukey's test ($p<0.05$).

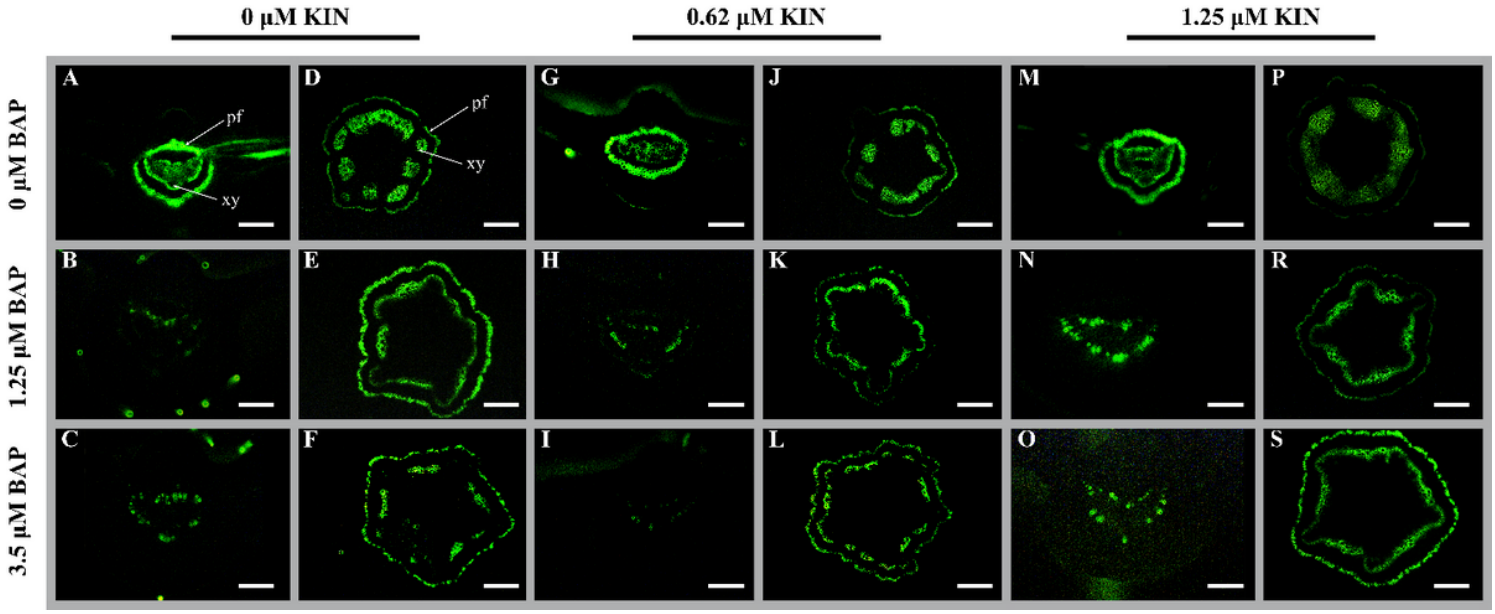

Figure 6

Stem and leaf cross-sections of *Q. robur* shoots grown in a medium with different concentrations of BAP (0, 1.25, and 3.5 µM) and KIN (0, 0.62, and 1.25 µM). Cross-sections stained by berberine hemi-sulfate and aniline blue solutions. pf – phloem fibers; xy – xylem. Bars = 100 µm (A-C, G-I, and M-O) and 200 µm (D-F, J-L, and P-S).

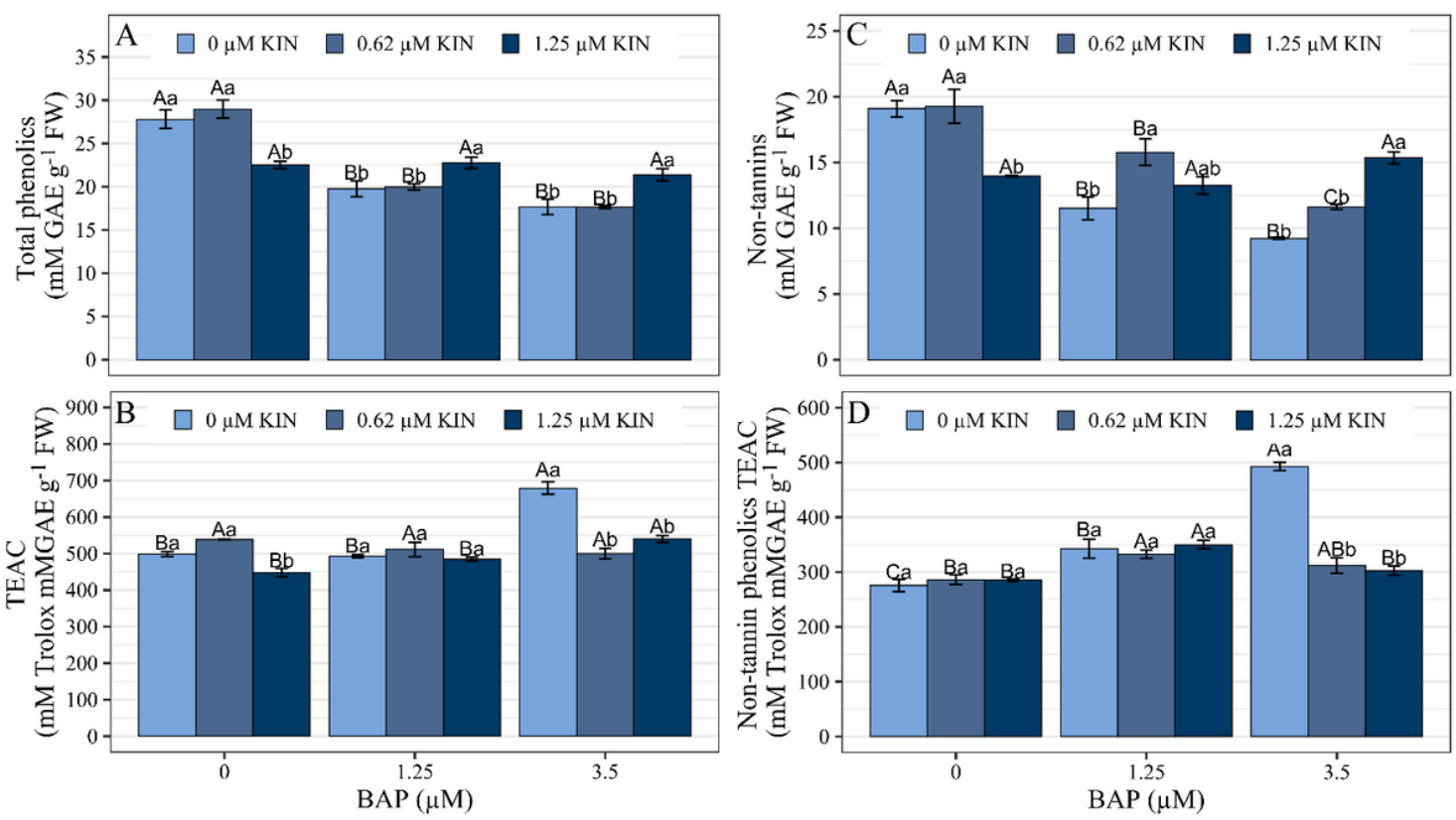

Figure 7

Phenolic content and antioxidant capacity of *Q. robur* leaves as a function of cytokinins (BAP, KIN) during *in vitro* culture. For each anatomical trait, means (± SE), $n = 5$, followed by the same letter (uppercase letters comparing BAP concentrations at each KIN concentration and lowercase letters comparing KIN concentrations at each BAP concentration), do not differ significantly according to Tukey's test ($p<0.05$).

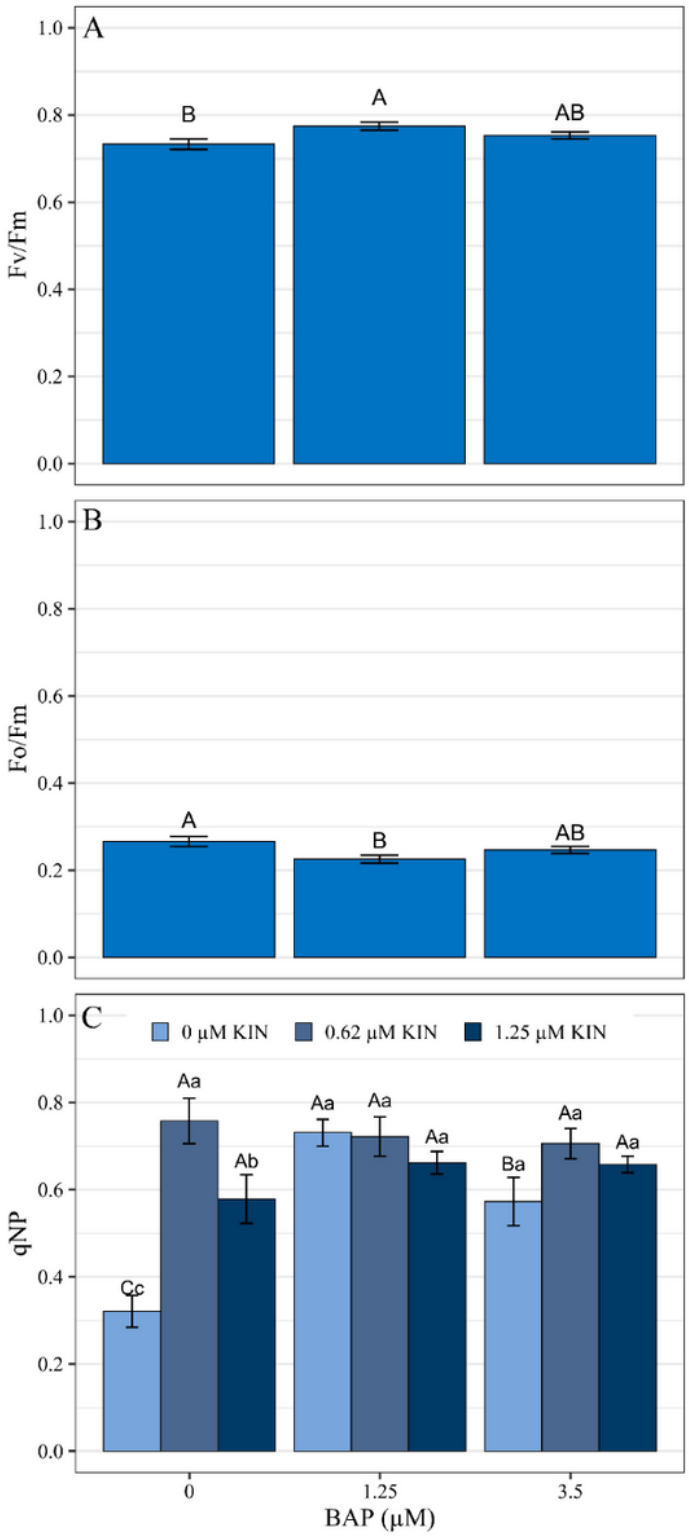

Figure 8

Parameters of modulated chlorophyll *a* fluorescence of *Q. robur* leaves as a function of cytokinins (BAP, KIN) during *in vitro* culture. For each parameter, means (± SE), $n = 6$, followed by the same letter (uppercase letters comparing BAP concentrations and lowercase letters comparing KIN concentrations at each BAP concentration), do not differ significantly according to Tukey's test ($p<0.05$).

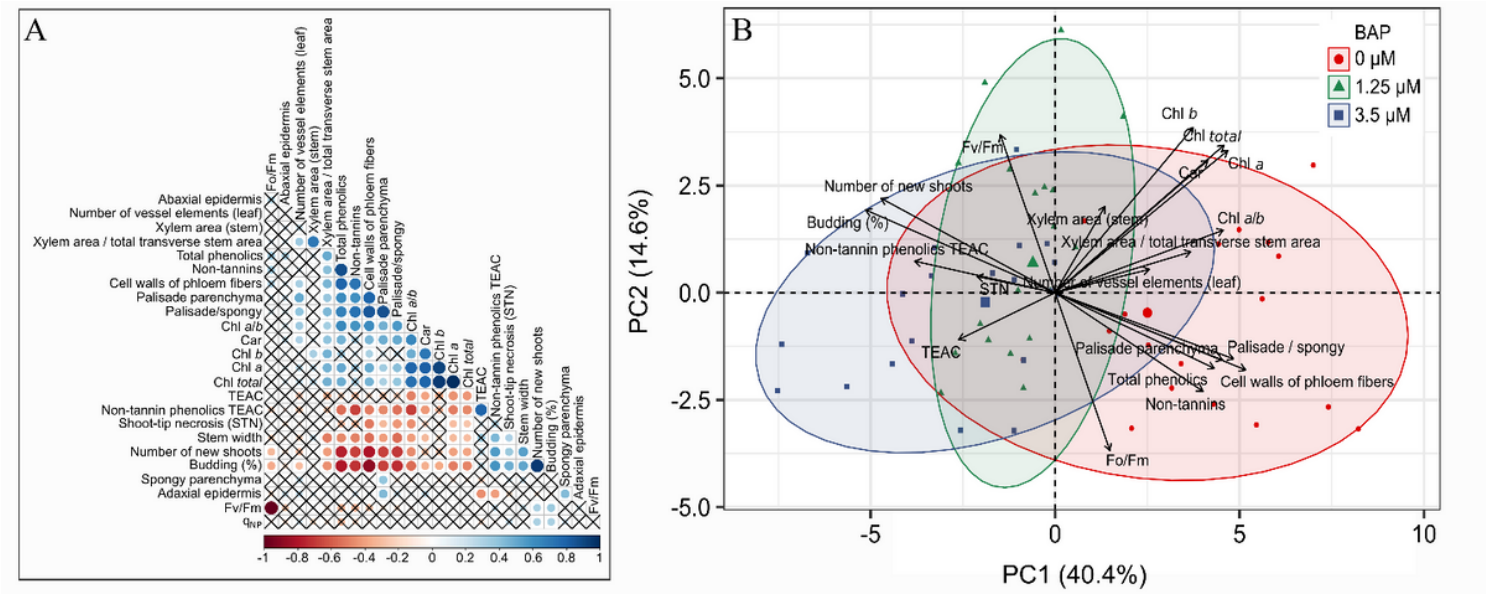

Figure 9

Pearson correlation coefficient and principal component analysis of growth, anatomical, and biochemical traits of *Q. robur* shoots as a function of cytokinin treatments during in vitro culture.