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Optimization of Adventitious Shoot Regeneration Protocols for Six *Vaccinium corymbosum* Cultivars

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Abstract. **Limitations in tissue culture and regeneration techniques present significant barriers to the development of improved blueberry (*Vaccinium* spp.) cultivars, restricting the adoption of tissue culture in commercial production. This challenge emphasizes the urgent need for a standardized regeneration protocol. Addressing this, we developed enhanced in vitro regeneration protocols for six highbush blueberry (*Vaccinium corymbosum* L.) cultivars—Emerald, NC5288, Rowan (NC3104), Legacy, Pinnacle, and Jewel—each with varying levels of tissue culture recalcitrance. We evaluated the effects of auxins, specifically α-Naphthaleneacetic Acid, cytokinins [including trans-Zeatin (ZT), 1-(2-Chloro-4-pyridyl)-3-phenylurea 98.0+%, TCI America™ (CPPU), and 6-γ-γ-[Dimethylallylamino]-purine (2iP)], and Thidiazuron (TDZ), which exhibits both auxin- and cytokinin-like activities, in various plant species cultured in vitro, on callus induction and regeneration from leaf explants. Media supplemented with ZT + 2iP (PZ9, PZ10) consistently outperformed all other treatments, enhancing regeneration significantly across all cultivars with high genotype-independent reproducibility, particularly in treatment PZ10. In contrast, TDZ-based treatments yielded highly variable results and were ineffective in recalcitrant cultivars such as Jewel and Pinnacle. ZT + CPPU treatments performed moderately across all metrics and may offer utility for cultivars with intermediate responsiveness. These findings provide critical insights into refining tissue culture techniques, offering a foundation for improving blueberry cultivar development and efficient genome editing. This work represents the first published report of in vitro regeneration for the highbush blueberry cultivars Rowan (NC3104), NC5288, and Pinnacle, expanding the scope of possibilities for advancing blueberry crop improvement.**

Blueberry (*Vaccinium* spp.) is a valuable, small fruit crop known for its polyphenol content, particularly anthocyanins, which have antioxidant, anti-inflammatory, antiobesity, and neuroprotective properties, driving significant demand (Cappai et al. 2018; Fang et al. 2020; Wu et al. 2023). Between 2015 and 2019, global blueberry export volumes grew by an average of 46,000 t annually. If current growth trends persist, the export value from key producers such as the United States, Peru, and Chile is projected to reach $3 billion by 2025 (US Department of Agriculture, Foreign Agricultural Service 2021). Despite its significance, blueberry propagation faces significant challenges in meeting the demand for high-quality plants. Conventional methods, including generative and vegetative propagation, are often inefficient and difficult to scale (Lobos and Hancock 2015; Mazurek et al. 2023). Generative reproduction, which produces genetically diverse seedlings through gamete fusion, is rarely used because of low seed yield, poor germination rates, and inconsistent seedling quality. Conversely, vegetative propagation, which involves rooting semi-woody shoots under high-humidity conditions, ensures genetic uniformity with the mother plant, but is slow, labor-intensive, dependent on environmental conditions, and limited by the availability of donor plants (Marino et al. 2014; Mazurek et al. 2023). In addition, because vegetative propagation results in clonal plants, some consider the lack of genetic diversity a disadvantage, as it may increase susceptibility to diseases, pests, and environmental changes. However, recent research suggests that fully clonal plants can maintain high levels of genetic diversity within local populations—comparable to that of outcrossing species—likely a result of the accumulation of somatic mutations and the absence of sexual reproduction (Huang et al. 2021). Despite this, in large-scale commercial cultivation, genetic uniformity remains advantageous for ensuring consistent fruit quality, yield, and predictable growth patterns, making vegetative propagation the preferred method for blueberry production.

Tissue culture presents an advanced approach to vegetative propagation, enabling rapid, year-round production of genetically uniform plants through techniques such as meristem proliferation and shoot organogenesis (Qiu et al. 2018). Unlike traditional methods, tissue culture is not constrained by seasonal limitations or the number of donor plants, making it highly scalable. However, its success is influenced by several biotic and abiotic factors, including genotype, explant type, culture media composition, and plant growth regulators (PGRs) (Long et al. 2022). As a result, significant variability in plant regeneration efficiencies has been observed, with many blueberry cultivars displaying limited success in tissue culture–based propagation (Liu et al. 2010; Marino et al. 2014). Despite these challenges, optimizing tissue culture protocols can revolutionize blueberry propagation, making large-scale, efficient plant production more feasible.

The absence of a standardized regeneration protocol presents a significant barrier toward the broader integration of blueberry tissue culture in commercial cultivation, especially for genotypes that display a limited response to tissue culture propagation. This recalcitrance has led to nurseries and growers rejecting the cultivation and promotion of what would otherwise be outstanding cultivars. In addition, substantial resources are required for testing various media for blueberry tissue culture and regeneration, emphasizing the need to address these challenges so that greater resource allocation can be directed toward scaling up plant production.

Moreover, the pursuit of new cultivars capable of meeting the rigorous demands of the fresh-market industry has prompted the exploration of genetic engineering and editing tools, such as CRISPR, for enhancing blueberry traits (Ku and Ha 2020). A critical factor for applying genetic transformation and editing in blueberries successfully is to establish a reliable

regeneration system, given that shoot organogenesis serves as an essential step for genetic transformation. The limited number of reports documenting the successful genetic transformation and editing of blueberries highlights the urgent need for a comprehensive and efficient approach to overcome the existing bottlenecks (Han et al. 2022; Song and Sink 2004).

We evaluated the effectiveness of 10 distinct regeneration treatments (TN1–PZ10) across six highbush blueberry cultivars by testing unique combinations and concentrations of PGRs, including Thidiazuron (TDZ; product ID: T888; PhytoTechnology, Shawnee Mission, KS, USA), α-Naphthaleneacetic Acid (NAA; product ID: N600; PhytoTchnology), 1-(2-Chloro-4-pyridyl)-3-phenylurea 98.0+%, TCI America™ (CPPU; Tokyo Chemical Industry, Portland, OR, USA), 6-γ-γ-[Dimethylallylamino]-purine (2iP; bioWORLD, Dublin, OH, USA), and trans-Zeatin (ZT; product ID: Z125; PhytoTechnology), to advance blueberry regeneration and explore innovative propagation approaches for diverse cultivars. Among the highbush blueberry cultivars tested were Rowan (NC3104), NC5288, and Pinnacle, for which successful regeneration has not been reported previously. In our study, tremendous success was achieved using a media supplemented with ZT + 2iP (treatments PZ9 and PZ10) through the process of indirect shoot organogenesis. Our results show improved tissue culture and regeneration methods applicable to a broader array of blueberry cultivars, and lay the groundwork for potential advancements in genetic engineering and genome editing endeavors, including the potential to shorten substantially the typically lengthy plant production cycle.

Materials and Methods

Plant materials and culture media. Six in vitro–cultured *Vacccinium corymbosum* L. cultivars, each representing different degrees of tissue culture recalcitrance, were used in our study. These included 'Rowan' and 'NC5288', advanced selections from North Carolina State University known for their high-quality berries and successful in-house in vitro culture. Also included were 'Legacy' and 'Emerald', which have been reported as being responsive to shoot regeneration on different media (Liu et al. 2010; Song and Hancock 2012). In addition, our study included 'Jewel' and 'Pinnacle', two cultivars known for their recalcitrance to tissue culture propagation. Previous attempts by the North Carolina State University Micropropagation and Repository Unit and PhyllaTech, LLC, to establish these cultivars in culture were unsuccessful (Phillips W, personal communication).

In vitro cultures were initially established from greenhouse-grown meristem explants, which were cultured on Lloyd & McCown Woody Plant Basal Mixture (WPM; product ID: L154; PhytoTechnology) (McCown and Lloyd 1981) supplemented with Murashige & Skoog (MS) Vitamin Solution (1000×) (product ID: M553; PhytoTechnology) (Murashige and Skoog 1962), additional Thiamine HCL (product ID: T390; PhytoTechnology) (276.85 μM) and 0.01% myo-inositol (product ID: 1703; Phytotechnology), 0.02% Ferric Sodium EDDHA (FeNa-EDDHA) (Sequestrene 138; product ID: E349; PhytoTechnology), 0.15% 2-(N-morpholino) ethanesulfonic acid (MES; product ID: M825; PhytoTechnology), 18.2 μM ZT, 2.0% D-Sucrose (product ID: S829; PhytoTechnology), and 0.6% Agar (product ID: A175; PhytoTechnology) at a pH of 5.0. After adventitious shoot formation, shoots were moved to Magenta™ vessel GA-7 plant culture boxes (Sigma Chemical Co., St. Louis, MO, USA) to be cultured on blueberry proliferation media containing WPM supplemented with MS vitamins, additional Thiamine (276.85 μM) and 0.01% myo-inositol, 0.02% Sequestrene 138, 0.15% MES, 9.12 μM ZT, 2.0% D-Sucrose, and 0.6% Agar at a pH of 5.0. In vitro plantlets were maintained under a 16-/8-h light/dark cycle and a 40-$\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity or dark conditions at 25 ± 1 °C, and were subcultured monthly until well-developed leaf blades from auxiliary shoots were obtained for use as explants.

To optimize the regeneration media for a broader range of blueberry cultivars, WPM supplemented with MS vitamins, 2.0% D-Sucrose, and 0.6% Agar at a pH of 5.0 (Song and Sink 2004, 2006) was selected as the basal regeneration medium. This medium was supplemented further with treatments TN1 through PZ10 (Table 1). Treatments labeled TN contained 0.045, 2.27, or 4.45 μM TDZ and 2.69 μM NAA, whereas those labeled TNZ included an additional 18.2 μM ZT. The concentration of 2.69 μM NAA and 18.2 μM ZT was selected based on its proven ability to achieve high regeneration frequencies in previous studies (Cappelletti et al. 2016; Liu et al. 2010; Song and Sink 2004, 2006). TDZ (Ghosh et al. 2018) concentrations were chosen for their documented success in media containing 4.45 μM TDZ (Liu et al. 2010), which was identified as the only treatment capable of regenerating the cultivar Jewel after five subcultures. Lower TDZ concentrations (0.0023, 0.91, and 2.27 μM) have been reported to promote in vitro proliferation and callus formation (Cappelletti et al. 2016; Liu et al. 2010). In contrast, a greater concentration of 9.08 μM TDZ resulted in no regeneration in any tested cultivars (Liu et al. 2010). Based on these findings, we tested 4.45 μM TDZ (treatments TN1 and TNZ2) alongside two lower concentrations: 2.27 μM (treatments TN3 and TNZ4) and 0.045 μM (treatments TN5 and TNZ6).

Treatments labeled CZ and PZ combined 18.2 μM ZT with either CPPU or 2iP, respectively. Previous research demonstrated that CPPU at 8.07 and 12.1 μM was more effective than ZT alone in promoting shoot regeneration from blueberry leaf sections (Liu et al. 2010). Similarly, 2iP at 4.92 μM was shown to outperform ZT in inducing shoots, buds, and meristematic nodules in leaves of blueberry cultivars Sunrise, Duke, and Georgia Gem (Liu et al. 2010). For the cultivar Duke, the inclusion of 2iP in the media has also shown potential for addressing challenges related to shoot regeneration and elongation in more recalcitrant blueberry cultivars (Cappelletti et al. 2016). Furthermore, a combination of 9.12 μM ZT and 24.6 μM 2iP has been reported to achieve regeneration frequencies ranging from 43.3% to 100% in cultivars such as Brigitta and Elliot (Song and Sink 2004). Building on these findings, we tested ZT in combination with CPPU at 8.07 μM (treatment CZ7) and 12.1 μM (treatment CZ8), as well as 2iP at 2.46 μM (treatment PZ9) and 4.92 μM (treatment PZ10). This approach allowed us to investigate whether ZT and CPPU combinations could replicate or improve on the regeneration outcomes observed with ZT and 2iP (Song and Sink 2004).

Callus induction and adventitious shoot regeneration. Approximately 4- to 8-mm-long leaf explants from in vitro shoots of highbush blueberry cultivars Emerald, NC5288, Rowan, Legacy, Pinnacle, and Jewel were dissected carefully from the stem. The proximal portion of the leaf blade was then positioned with the abaxial side facing downward on 30 mL of regeneration media, supplemented with individual treatments TN1 through PZ10 (Table 1) and adjusted to a pH of 5.0, with each treatment consisting of two replicates, and each replicate comprising three plates. Because of the availability of in vitro plantlets, plates for 'Rowan' and 'Legacy' contained 10 leaf explants each, whereas plates for cultivars

Table 1. Plant growth regulator combinations evaluated for optimized regeneration media in six *V. corymbosum* L. cultivars with varying tissue culture recalcitrance.

	Plant growth regulator (μM)				
Treatment	TDZ	NAA	ZT	CPPU	2iP
TN1	0.045	2.69	—	—	—
TNZ2	0.045	2.69	18.2	—	—
TN3	2.27	2.69	—	—	—
TNZ4	2.27	2.69	18.2	—	—
TN5	4.45	2.69	—	—	—
TNZ6	4.45	2.69	18.2	—	—
CZ7	—	—	18.2	8.07	—
CZ8	—	—	18.2	12.1	—
PZ9	—	—	18.2	—	2.46
PZ10	—	—	18.2	—	4.92

2iP = 6-γ-γ-[Dimethylallylamino]-purine; CPPU = 1-(2-chloro-4-pyridyl)-3-phenylurea; NAA = α-Naphthaleneacetic Acid; TDZ = Thidiazuron; ZT = trans-Zeatin.

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Emerald, NC5288, Pinnacle, and Jewel contained five leaf explants each. The plates were initially kept in the dark at a temperature of 25 ± 1 °C for 2 weeks, followed by a transfer to an incubation environment with cool-white fluorescent lights, also maintained at 25 ± 1 °C under a 16-/8-h light/dark cycle and a 40-μmol·m^{-2}·s^{-1} light intensity or dark conditions. All treatments for all cultivars were initiated concurrently, and data collection was performed concomitantly across the same set of cultures to ensure consistency in scoring.

After a 2-, 4-, and 8-week culture period, data were collected on callus induction rate (percentage of the number of explants with callus in relation to the total number of explants cultured per petri dish), adventitious shoot regeneration frequency (number of explants with at least one shoot in relation to the number of explants cultured per petri dish), mean shoot numbers per explant, and callus-to-shoot conversion efficiency (percentage of the formed callus that resulted in shoot regeneration), including plate averages. After 8 weeks of culture and data collection, regenerated explants with shoots measuring at least 0.5 inch in length were transferred to plant culture boxes (Magenta™ vessel GA-7) containing blueberry proliferation media for continued culture at 25 ± 1 °C under a 16-/8-h light/dark cycle and a 40-μmol·m^{-2}·s^{-1} light intensity or dark conditions.

Data analysis. The data collected from the regeneration process underwent a comprehensive analysis to assess the effectiveness of 10 treatments in promoting callus formation and supporting the subsequent growth of adventitious shoots and explants. This evaluation was conducted at 2, 4, and 8 weeks of culture. However, because of contamination, data from the cultivars Pinnacle and Jewel were excluded from analysis beyond the 4-week mark. Consequently, the 8-week dataset included only 'Emerald', 'Rowan', 'NC5288', and 'Legacy'.

To facilitate accurate comparisons, the means and standard errors of the means of biological replicates were calculated using RStudio v. 4.4.2 (Posit Software, PBC, Boston, MA, USA). Data normalization was performed by averaging values per explant for each plate, accounting for differences in the number of leaf explants per plate among cultivars Legacy and Rowan (10 leaf explants per plate), and cultivars Emerald, NC5288;, Jewel, and Pinnacle (five leaf explants per plate).

The normality of data were evaluated using the Shapiro-Wilk test, which returned P values indicating deviations from normality ($P < 0.05$), suggesting that parametric test assumptions were violated. Attempts to normalize the data using log and square root transformations did not improve normality sufficiently. Therefore, nonparametric statistical methods were applied for subsequent analyses.

To compare treatment effects within each cultivar, and cultivar responses across treatments, the Kruskal-Wallis test was used as a nonparametric alternative to analysis of variance. This rank-based test determined whether significant differences existed in callus induction, shoot formation, shoot number per explant, and callus-to-shoot conversion efficiency among treatments. When significant differences were detected ($P < 0.05$), Dunn's test with Bonferroni-adjusted P values was performed for post hoc pairwise comparisons to control for multiple testing.

All statistical analyses were conducted in RStudio v. 4.4.2 (Posit Software, PBC, Boston, MA, USA), with statistical significance set at $P < 0.05$.

Results

The effects of PGRs on callus induction. Using leaf explants from in vitro shoots, we evaluated the effects of TDZ (0.045, 2.27, and 4.45 μM) and ZT (18.2 μM) in combination with NAA (2.69 μM), ZT (18.2 μM) with CPPU (8.07 and 12.1 μM), and ZT (18.2 μM) with 2iP (2.46 and 4.92 μM) on callus induction from six highbush blueberry cultivars: Emerald, NC5288, Rowan, Legacy, Pinnacle, and Jewel. Callus induction rates varied significantly across treatments in most cultivars, ranging from 10.0% to 65.0% under TDZ-based treatments (TN1–TNZ6), 30.0% to 56.7% under ZT + CPPU (treatments CZ7 and CZ8), and 46.7% to 83.3% under ZT + 2iP (treatments PZ9 and PZ10) (Fig. 1, Table 1).

Kruskal-Wallis tests confirmed significant treatment effects in 'Emerald' ($P = 0.0006$), 'Legacy' ($P < 0.0001$), 'NC5288' ($P = 0.0408$), 'Rowan' ($P < 0.0001$), and 'Pinnacle' ($P < 0.0001$), whereas 'Jewel' showed no significant differences (Supplemental Table 2). Dunn's post hoc tests revealed that treatments PZ9 and PZ10 (ZT + 2iP) increased callus induction significantly compared with TDZ-based treatments (TN1–TNZ6) in 'Emerald', 'Legacy', and 'Rowan' ($P < 0.05$) (Fig. 1, Supplemental Table 3). Notably, a significant difference was observed in 'Legacy' between treatments TN1 and TNZ2, suggesting that TDZ enhances callus induction only when used at a relatively low concentration in combination with NAA and ZT ($P < 0.05$) (Fig. 1, Supplemental Table 3). In 'Pinnacle', both ZT + 2iP (treatments PZ9 and PZ10) and ZT + CPPU (treatments CZ7 and CZ8) induced significantly greater callus formation than TDZ-based treatments (Fig. 1, Supplemental Table 3). In addition, in 'NC5288', treatment PZ9 increased callus induction significantly compared with treatment TN3 (Fig. 1, Supplemental Table 3).

Genotypic responses to treatments varied more under TDZ-based treatments (TN1–TNZ6) compared with ZT + 2iP (treatments PZ9 and PZ10) and ZT + CPPU (treatments CZ7 and CZ8) (Fig. 2). Kruskal-Wallis tests confirmed significant genotype effects within treatments TN1 ($P = 0.0024$), TNZ2 ($P = 0.0002$), TN3 ($P = 0.0002$), TNZ4 ($P = 0.0057$), TN5 ($P = 0.0079$), TNZ6 ($P = 0.0037$), and PZ9 ($P = 0.0071$) (Supplemental Table 2). Dunn's post hoc tests showed significant genotype differences, particularly between 'Pinnacle' and most other cultivars under TDZ-based treatments (TN1–TNZ6), and between 'Emerald' and 'Jewel' as well as 'Emerald' and 'Pinnacle' under treatment PZ9 (Fig. 2, Supplemental Table 3).

Hierarchical clustering of callus induction rates revealed a clear separation between treatment groups (Fig. 3A). Treatments containing ZT + 2iP (treatments PZ9 and PZ10) clustered together and were strongly associated with greater callus induction rates (≥ 60.0%) across most cultivars, particularly in 'Emerald', 'Pinnacle', and 'Rowan'. In contrast, TDZ-based treatments (TN1–TNZ6) formed a distinct cluster characterized by lower callus induction rates, with treatments TN3, TN5, and TNZ6 showing the least callus formation across all cultivars. The ZT + CPPU treatments (CZ7 and CZ8) clustered separately, displaying intermediate callus induction rates ranging from 30.0% to 56.7% (Fig. 3A).

PGR impact on adventitious shoot regeneration. Using the same in vitro leaf explants, we next assessed the effects of treatments (TN1–PZ10) on shoot regeneration rates and mean shoot numbers across the six highbush blueberry cultivars. Both shoot regeneration and mean shoot production per explant varied significantly among treatments (Fig. 1). In TDZ + NAA treatments (TN1–TNZ6), shoot regeneration ranged from 3.3% to 65.0%, with an average of 0.1 to 2.3 shoots per explant (Fig. 1, Supplemental Table 1). Treatments containing ZT + CPPU (treatments CZ7 and CZ8) resulted in 10.0% to 36.7% shoot regeneration rates, producing 0.2 to 1.4 shoots per explant. When ZT was combined with 2iP (treatments PZ9 and PZ10), shoot regeneration ranged from 30.0% to 65.0%, with mean shoot numbers between 0.7 and 2.9, showing a broader range of response across cultivars (Fig. 1, Supplemental Table 1).

Kruskal-Wallis tests confirmed significant treatment effects for shoot regeneration and mean shoot number per explant across all cultivars ($P \leq 0.0001$) (Supplemental Table 2). Dunn's post hoc tests revealed that ZT + 2iP treatments (PZ9 and PZ10) increased shoot regeneration and mean shoot number per explant significantly compared with TDZ-based treatments (TN1–TNZ6) in 'Jewel', 'Pinnacle', 'Emerald', 'Rowan', and 'Legacy' ($P < 0.05$) (Fig. 1, Supplemental Table 3).

Under TDZ-based treatments (TN1–TNZ6), 'Jewel' and 'Pinnacle' failed to regenerate shoots entirely. At the same time, 'Emerald' showed no regeneration under treatments TN1, TN3, and TN5, which contained 0.045, 2.27, and 4.45 μM TDZ, respectively, plus NAA, as well as treatment TNZ2 (0.045 μM TDZ + NAA + ZT) (Fig. 1, Supplemental Table 1). 'Emerald' only regenerated shoots under treatments TNZ4 and TNZ6 (2.27 and 4.45 μM TDZ, respectively, + NAA + ZT), suggesting that TDZ promoted shoot regeneration in this cultivar only at greater concentrations and when combined with NAA and ZT (Fig. 1, Supplemental Table 1).

Conversely, 'Rowan' regenerated shoots only under treatments TN1 through TNZ4, which contained 0.045 and 2.27 μM TDZ, respectively, plus NAA and/or ZT, indicating the opposite trend—that TDZ promoted shoot regeneration in 'Rowan' only at lower

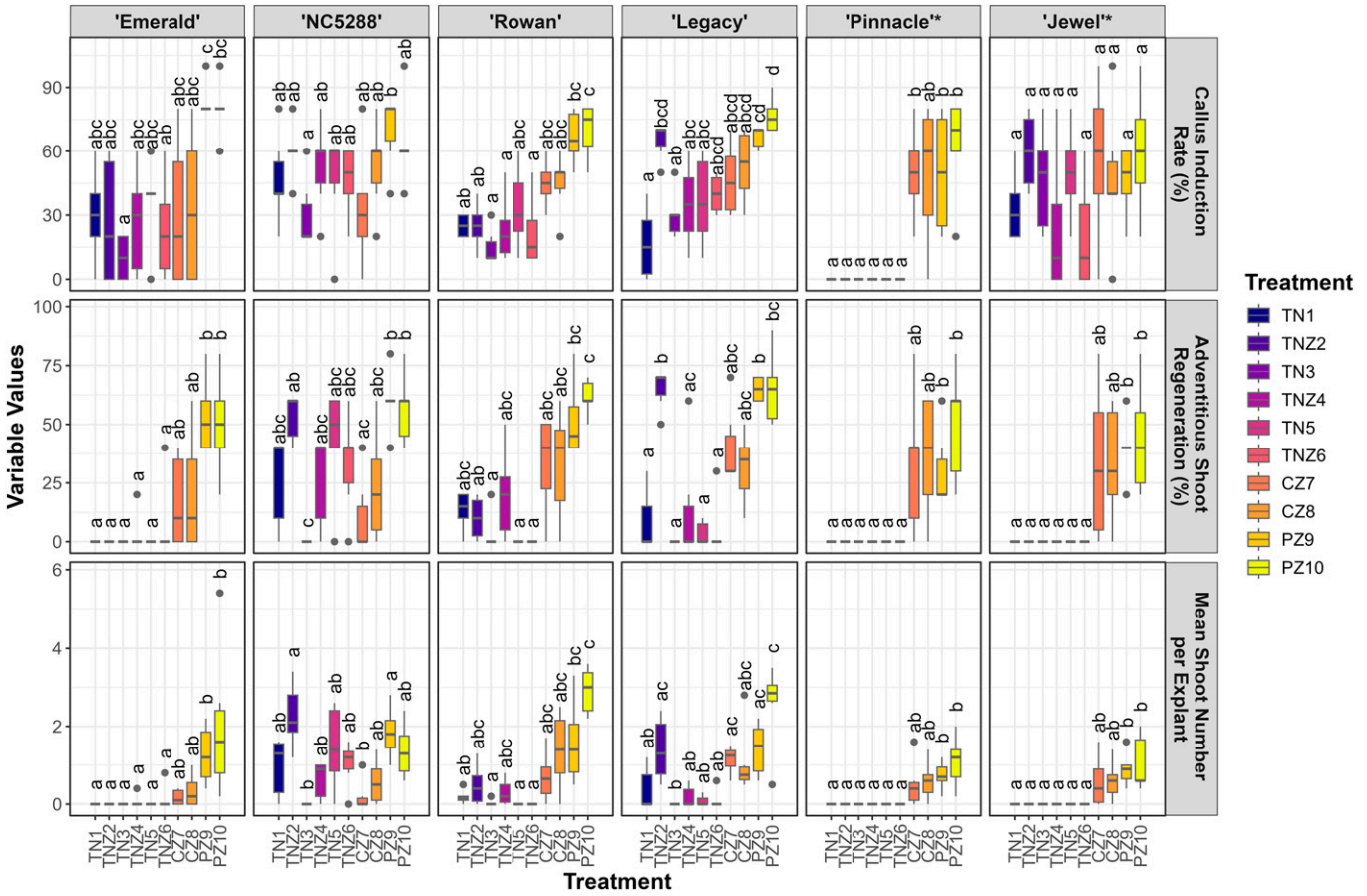

Fig. 1. Treatment comparisons within individual cultivars for callus induction rate (measured as a percentage), adventitious shoot regeneration frequency (measured as a percentage), and mean shoot number per explant in six *Vaccinium corymbosum* L. cultivars grown on woody plant medium–based regeneration media supplemented with treatments TN1 through PZ10. Boxes represent the interquartile range (25th–75th percentile), with horizontal lines indicating the median. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Dots represent outliers. The letters above boxplots indicate statistically significant differences among treatments determined by the Kruskal-Wallis test, followed by Dunn's post hoc test with Bonferroni-adjusted P values ($P < 0.05$). Treatments that share a letter are not significantly different. Data represent results after an 8-week culture period. * = observations made at the 4-week mark.

concentrations, regardless of ZT addition (Fig. 1, Supplemental Table 1). In 'Legacy', significant differences were observed between TDZ + NAA and TDZ + NAA + ZT treatments, particularly between TN1, TN3, and TN5 vs. TNZ2 ($P < 0.05$), as well as between TNZ2 and TNZ6 ($P < 0.05$). These results suggest that the addition of ZT to lower concentrations of TDZ + NAA influenced shoot regeneration responses (Fig. 1, Supplemental Table 3).

In 'NC5288', ZT + 2iP treatments (PZ9 and PZ10) increased shoot formation significantly compared with treatments CZ7 and TN3 ($P < 0.05$), indicating that TDZ-based treatments were more effective in this cultivar, closely resembling the performance of the best-performing ZT + 2iP treatments (PZ9 and PZ10) (Fig. 1, Supplemental Table 3). In addition, 'NC5288' exhibited lower shoot regeneration under the CZ7 treatment compared with treatments PZ9 and PZ10, reinforcing that cytokinin type played a critical role in shoot formation responses in this cultivar (Fig. 1, Supplemental Table 3).

Genotypic responses to shoot regeneration and mean shoot number were more variable under TDZ-based treatments (TN1–TNZ6) compared with ZT + 2iP (treatments PZ9 and PZ10) and ZT + CPPU (treatments CZ7 and CZ8) (Fig. 2). Kruskal-Wallis tests confirmed significant genotype effects for shoot regeneration within treatments TN1, TNZ2, TNZ4, TN5, TNZ6, and PZ9 ($P < 0.05$) and for mean shoot number per explant within treatments TN1, TNZ2, TNZ4, TN5, TNZ6, and PZ10 ($P < 0.05$) (Supplemental Table 2). Dunn's tests further identified significant differences, with 'NC5288' consistently exhibiting greater shoot regeneration and shoot numbers than most other cultivars under TDZ-based treatments (TN1–TNZ6) (Fig. 2, Supplemental Table 3).

Interestingly, genotypic variation was particularly evident in treatment PZ9 for shoot regeneration and treatment PZ10 for mean shoot number, suggesting that cytokinin concentration may influence the pattern of shoot formation. Shoot regeneration typically follows two distinct patterns: pattern I, where shoots emerge primarily at wound sites, and pattern II, which is characterized by uniform shoot formation across the explant surface (Liu et al. 2010). Under lower 2iP concentrations (2.46 μM), 'Emerald' and 'Rowan' exhibited predominantly pattern II regeneration, whereas greater 2iP concentrations (4.92 μM) induced pattern I (Fig. 4A1, A2, B1, and B2). In contrast, 'Pinnacle' responded inversely, displaying pattern I at lower concentrations and pattern II at greater concentrations (Fig. 4E1 and E2). 'NC5288', 'Legacy', and 'Jewel' consistently followed pattern I regeneration across both 2iP concentrations (Fig. 4E1, E2, D1, D2, F1, and F2, respectively).

Hierarchical clustering revealed a clear distinction between treatment groups for both adventitious shoot regeneration and mean shoot number per explant (Fig. 3B and C). For shoot regeneration, ZT + 2iP treatments (PZ9 and PZ10) clustered together consistently and were strongly associated with the greatest regeneration rates, particularly in 'Rowan', 'Emerald', and 'Pinnacle' (≥ 50.0%) (Fig. 3B). In most cultivars, TDZ-based treatments (TN1–TNZ6) formed a separate cluster, exhibiting consistently low regeneration percentages. However, treatment TNZ2 grouped with ZT + 2iP in 'Legacy' and 'NC5288', achieving regeneration rates of ≥ 50.0% (Fig. 3B). Meanwhile, ZT + CPPU treatments (CZ7 and CZ8) formed an intermediate cluster, with moderate shoot regeneration rates ranging from 10.0% to 40.0% (Fig. 3B).

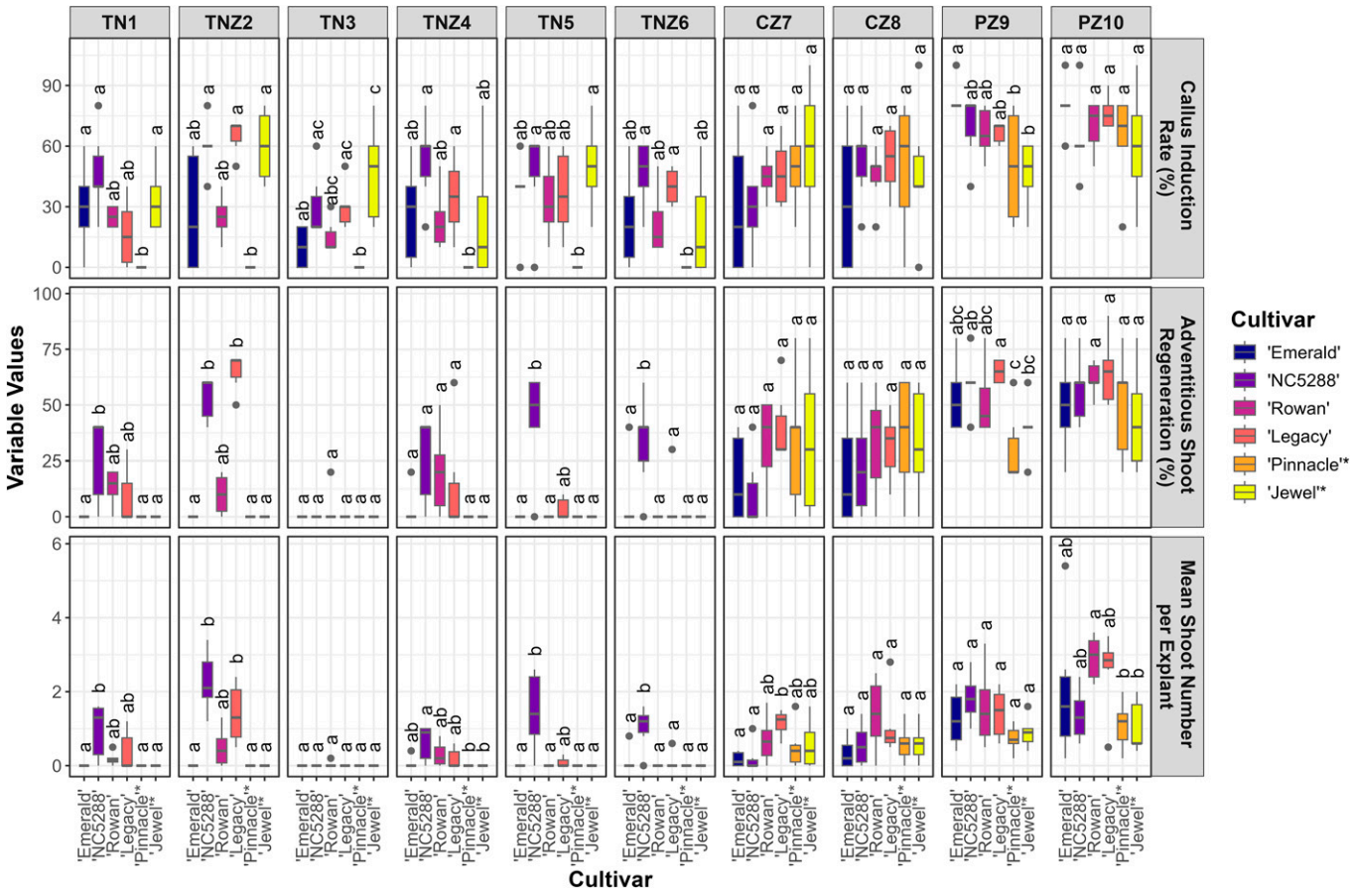

Fig. 2. Cultivar comparisons within a single treatment for callus induction rate (measured as a percentage), adventitious shoot regeneration frequency (measured as a percentage), and mean shoot number per explant in six *Vaccinium corymbosum* L. cultivars grown on woody plant medium–based regeneration media supplemented with treatments TN1 through PZ10. Boxes represent the interquartile range (25th–75th percentile), with horizontal lines indicating the median. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Dots represent outliers. The letters above boxplots indicate statistically significant differences among treatments determined by the Kruskal-Wallis test, followed by Dunn's post hoc test with Bonferroni-adjusted *P* values ($P < 0.05$). Treatments that share a letter are not significantly different. Data represent results after an 8-week culture period. * = observations made at the 4-week mark.

For mean shoot number per explant, ZT + 2iP (treatments PZ9 and PZ10) again clustered separately, showing the highest shoot numbers across most cultivars, particularly in 'Rowan' (2.92 shoots per explant), 'Legacy' (2.57 shoots per explant), and 'Emerald' (2.00 shoots per explant) (Fig. 3C). 'NC5288' and 'Pinnacle' exhibited lower shoot numbers, averaging 1.37 and 1.10 shoots per explant, respectively (Fig. 3C).

In contrast, TDZ-based treatments (TN1–TNZ6) clustered separately with consistently low shoot numbers across all cultivars, generally < 1.00, with most cultivars showing minimal or no shoot production (Fig. 3C). 'NC5288' displayed slightly higher shoot numbers than other cultivars under TDZ-based treatments, though still at low levels (Fig. 3C). Meanwhile, ZT + CPPU treatments (CZ7 and CZ8) occupied an intermediate cluster, producing moderate shoot numbers (1.00–2.27 shoots per explant), particularly in 'NC5288' and 'Emerald' (Fig. 3C). Although these treatments improved shoot production compared with TDZ-based treatments, they did not reach the levels observed under ZT + 2iP.

These findings reinforce ZT + 2iP as the most effective cytokinin combination for promoting both shoot regeneration and shoot proliferation, whereas TDZ-based treatments consistently resulted in poor performance across cultivars.

Influence of PGRs on callus-to-shoot conversion efficiency. Except for 'Pinnacle', which failed to induce callus under TDZ-based treatments (TN1–TNZ6), successful callus induction was observed in all other genotypes (Fig. 1). However, callus induction did not result in increased shoot production consistently (Fig. 1). Notably, shoot production across all six cultivars was only stimulated after 2 weeks of culture when ZT was combined with 2iP (Supplemental Table 1). This trend persisted over more extended culture periods, as many calli failed to regenerate adventitious shoots even after 4 or 8 weeks (Supplemental Table 1).

To assess the relationship between callus formations and shoot regeneration, we analyzed callus-to-shoot conversion efficiency, which is defined as the percentage of calli that regenerated shoots successfully. Conversion rates ranged from 11.1% to 100% under TDZ-based treatments (TN1–TNZ6), from 27.8% to 66.7% under ZT + CPPU (treatments CZ7 and CZ8), and from 65.0% to 97.6% under ZT + 2iP (treatments PZ9 and PZ10) (Fig. 5, Supplemental Table 1).

Kruskal-Wallis tests confirmed significant treatment effects on callus-to-shoot conversion efficiency across all cultivars ($P < 0.01$, Supplemental Table 2). Dunn's post hoc tests further revealed that ZT + 2iP treatments (PZ9 and PZ10) improved conversion efficiency significantly compared with multiple TDZ-based treatments (TN1–TNZ6) in 'Emerald', 'Jewel', and 'Rowan' ($P < 0.05$, Supplemental Table 3).

'Pinnacle', which failed to form callus or regenerate shoots under TDZ-based treatments (TN1–TNZ6), was excluded from further analysis, as no significant differences in conversion efficiency were observed under the remaining ZT + 2iP (treatments PZ9 and PZ10) and ZT + CPPU (treatments CZ7 and CZ8) treatments (Fig. 5, Supplemental Table 3).

In 'Emerald', the conversion efficiency was significantly greater under treatments PZ9 and PZ10 compared with treatments TN1, TN3, TN5, and TNZ2 ($P < 0.01$), all of which failed to regenerate shoots from callus (Fig. 5, Supplemental Table 3). A similar trend was observed in 'Jewel', where treatments PZ9 and PZ10 outperformed all TDZ-based treatments

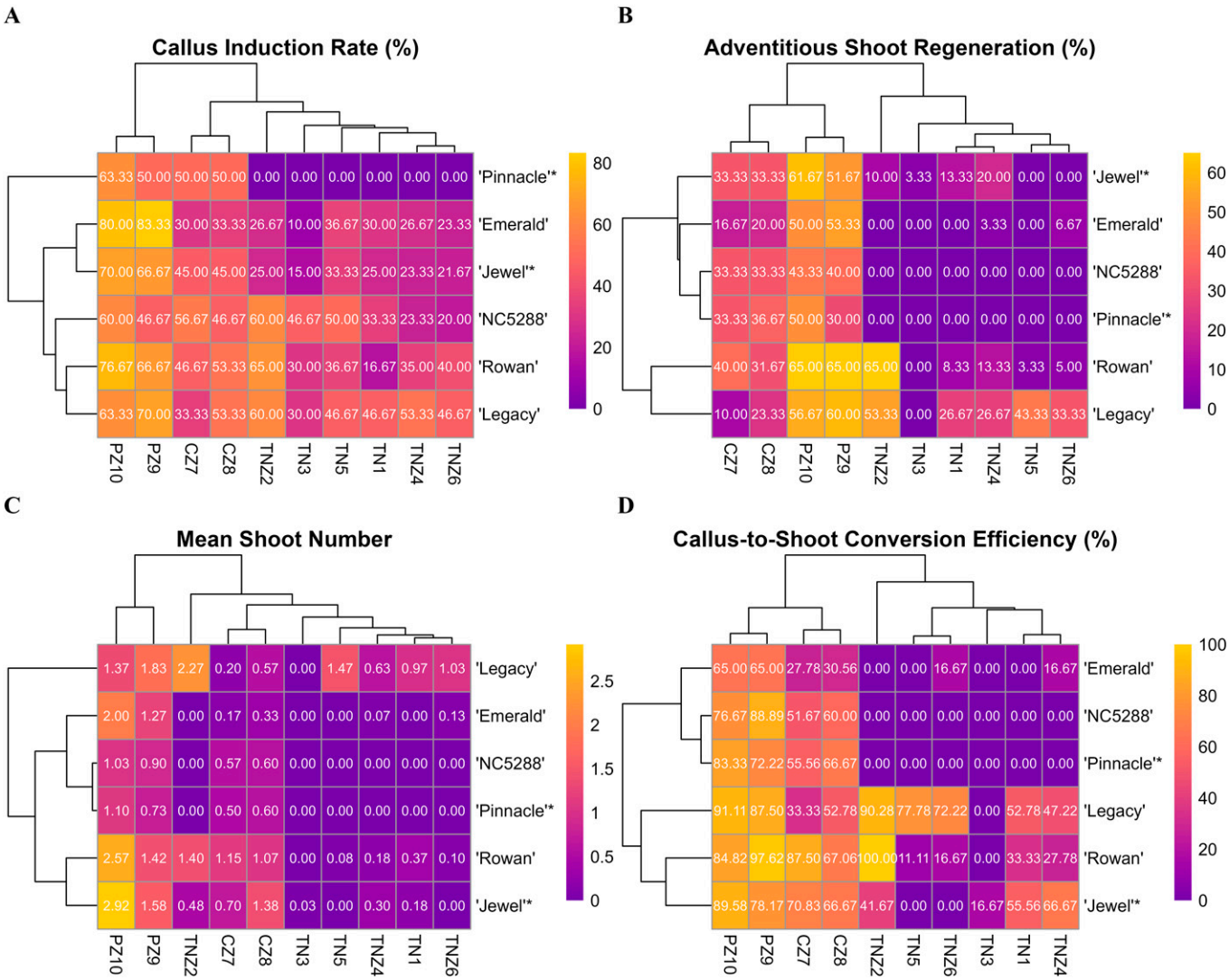

Fig. 3. Heatmap visualization of callus induction rate (**A**) (measured as a percentage, adventitious shoot regeneration frequency (**B**) (measured as a percentage), mean shoot number per explant (**C**), and callus-to-shoot conversion efficiency (**D**) (measured as a percentage) in six *Vaccinium corymbosum* L. cultivars grown on woody plant medium–based regeneration media supplemented with treatments TN1 through PZ10. Rows represent cultivars, columns represent treatments, and hierarchical clustering groups similar responses based on treatment effects. Color gradients range from purple (low values) to yellow (high values), illustrating the variation in regeneration efficiency across cultivars and treatments. Data represent results after an 8-week culture period. * = observations made at the 4-week mark.

significantly ($P < 0.01$), which were also unable to regenerate shoots from the callus (Fig. 5, Supplemental Table 3). In 'Legacy', treatments PZ9 improved conversion efficiency significantly over the unresponsive treatments TN3 ($P = 0.0073$) and ($P = 0.0437$), whereas treatment CZ7 (ZT + CPPU) also performed better than treatment TN3 ($P = 0.0304$) (Fig. 5, Supplemental Table 3). In 'NC5288', treatments PZ9 and PZ10 increased conversion efficiency significantly compared with treatment TN3, which failed to regenerate shoots ($P < 0.05$, Fig. 5, Supplemental Table 3). Last, in 'Rowan', treatment PZ10 outperformed treatments TN5 and TNZ6 significantly ($P < 0.05$), confirming its effectiveness in promoting shoot conversion (Fig. 5, Supplemental Table 3).

Intriguingly, treatment TN3 induced callus formation in five of the six cultivars. Yet, only 'Rowan' regenerated shoots, and even then at a low rate of 16.7% (Fig. 5). In addition, although TDZ-based treatments (TN1–TNZ6) induced callus formation successfully in 'Emerald' and 'Jewel', none except treatment TNZ4 in 'Emerald' (16.7% conversion) produced any shoots (Fig. 5). These findings highlight the limited effectiveness of TDZ-based treatments for shoot regeneration in the tested cultivars.

Genotypic responses to callus-to-shoot conversion efficiency were more variable under TDZ-based treatments (TN1–TNZ6) than under ZT + 2iP (treatments PZ9 and PZ10) or ZT + CPPU (treatments CZ7 and CZ8) (Supplemental Table 2). Kruskal-Wallis tests confirmed significant genotype effects within treatments TN1 ($P = 0.0047$), TNZ2 ($P < 0.0001$), TNZ4 ($P = 0.0289$), TN5 ($P = 0.0003$), and TNZ6 ($P = 0.0025$), whereas no significant cultivar differences were observed in ZT + CPPU (treatments CZ7 and CZ8) or ZT + 2iP (treatments PZ9 and PZ10) (Supplemental Table 2).

Hierarchical clustering revealed that ZT + 2iP treatments (treatments PZ9 and PZ10) formed a distinct group associated with the highest callus-to-shoot conversion efficiencies across all cultivars (Fig. 3D). Within this group, 'Jewel', 'Pinnacle', 'NC5288', 'Legacy', and 'Rowan' exhibited the greatest conversion rates (≥ 72.2%), whereas 'Emerald' showed slightly lower efficiency (65.0%) (Fig. 3D).

In contrast, TDZ-based treatments (TN1–TNZ6) clustered separately and were consistently associated with low or zero conversion efficiency across all cultivars. Notably, treatment TNZ2 showed slightly greater conversion rates in 'NC5288' and 'Legacy', but remained distinct from the ZT + 2iP cluster (Fig. 3D). Meanwhile, ZT + CPPU treatments (CZ7 and CZ8) occupied an intermediate position, with conversion rates ranging from 27.8% to 87.5%, depending on the cultivar (Fig. 3D). These findings reinforce the superior effectiveness of ZT + 2iP in promoting

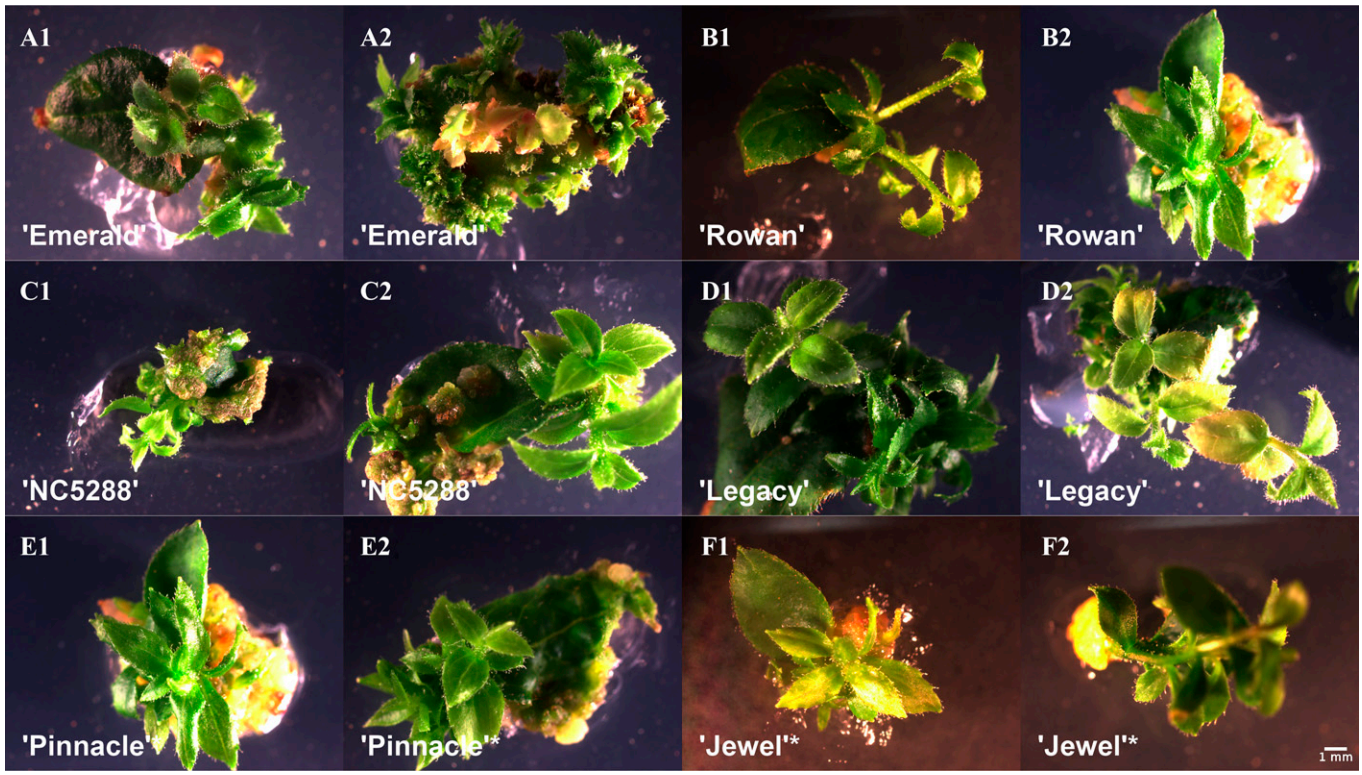

Fig. 4. Adventitious shoot regeneration from highbush blueberry cultivars cultured on woody plant medium–based regeneration media supplemented with trans-Zeatin and 6-γ-γ-[Dimethylallylamino]-purine. (**A1**) Treatment PZ9 pattern I shoot regeneration in 'Emerald'. (**A2**) Treatment PZ10 pattern II shoot regeneration in 'Emerald'. (**B1**) Treatment PZ9 pattern I shoot regeneration in 'Rowan'. (**B2**) Treatment PZ10 pattern I shoot regeneration in 'Rowan'. (**C1**) Treatment PZ9 pattern II shoot regeneration in 'NC5288'. (**C2**). Treatment PZ10 pattern II shoot regeneration in 'NC5288'. (**D1**) Treatment PZ9 pattern I shoot regeneration in 'Legacy'. (**D2**) Treatment PZ10 pattern I shoot regeneration in 'Legacy'. (**E1**) Treatment PZ9 pattern I shoot regeneration in 'Pinnacle'. (**E2**) Treatment PZ10 pattern I shoot regeneration in 'Pinnacle'. (**F1**) Treatment PZ9 pattern I shoot regeneration. (**F2**) Treatment PZ10 pattern I shoot regeneration. Images represent results after an 8-week culture period. * = observations made at the 4-week mark. Images taken at ×10 magnification. Scale bar = 1 mm.

callus-to-shoot conversion across multiple cultivars, and further emphasizes the limited regenerative potential of TDZ-based treatments.

Discussion

Tissue culture has long been explored as a method for improving blueberry propagation; however, its success remains highly genotype dependent, limiting its widespread application in commercial production. Many highbush cultivars exhibit recalcitrance to in vitro propagation, requiring extensive optimization for each genotype. This challenge highlights the need for a versatile and efficient regeneration protocol capable of supporting shoot organogenesis across a broader range of cultivars.

To address this limitation, we developed and tested 10 treatments (TN1–PZ10) to evaluate the effects of various PGR combinations and concentrations on callus induction, shoot regeneration, mean shoot number per explant, and callus-to-shoot conversion efficiency. Our findings demonstrate that ZT + 2iP treatments (PZ9 and PZ10) consistently outperformed other PGR combinations, improving all tested parameters across multiple cultivars significantly (Fig. 3A–D). In contrast, TDZ-based treatments (TN1–TNZ6) exhibited strong genotype-dependent responses, often yielding variable and ineffective results. This was particularly evident in recalcitrant cultivars such as Jewel and Pinnacle, which failed to regenerate shoots entirely under TDZ-containing media (Fig. 5). Meanwhile, ZT + CPPU treatments (CZ7 and CZ8) also performed well, though their responses were generally intermediate. Depending on the cultivar, these treatments tended to cluster separately, displaying moderate callus induction rates, shoot regeneration frequencies, mean shoot numbers, and callus-to-shoot conversion efficiencies (Fig. 4A–D). Their intermediate performance suggests they may be valuable in specific genotypic contexts or as supplements to other optimized protocols.

'NC5288' and 'Legacy' stood out for their adaptability to TDZ-based treatments among more responsive cultivars. 'NC5288' showed consistently greater shoot regeneration and shoot numbers under these treatments compared with other cultivars (Fig. 3B and C, Supplemental Table 3). Both 'NC5288' and 'Legacy' achieved shoot regeneration rates of ≥ 50.0% under treatment TNZ2, which clustered with the high-performing ZT + 2iP treatments (PZ9 and PZ10), reinforcing their broader responsiveness to diverse PGR formulations (Fig. 3B). These findings suggest that although TDZ can be effective for specific genotypes, its application requires precise and cultivar-specific optimization, making it less reliable for standardizing blueberry regeneration protocols.

PGR concentration within TDZ-based treatments also played a critical role in shoot response. 'Emerald' only regenerated shoots when TDZ was applied at greater concentrations (2.27 and 4.45 μM) and when combined with NAA and ZT, whereas 'Rowan' responded best to lower TDZ concentrations (0.045 μM), regardless of ZT addition (Fig. 1). In 'Legacy', significant differences between TDZ + NAA and TDZ + NAA + ZT treatments suggest that ZT modulates the effect of TDZ at lower concentrations (Fig. 1, Supplemental Table 3). These results further underscore the genotype-specific effects of TDZ and the importance of tailored PGR combinations.

This study highlights the high reproducibility and genotype-independent performance of treatment PZ10 (ZT + 4.92 μM 2iP). Treatment PZ10 showed no significant variation among cultivars across callus induction, shoot regeneration, and callus-to-shoot conversion, making it a strong candidate for a standardized regeneration medium (Supplemental Table 2). Treatment PZ9 (ZT + 2.46 μM 2iP) also performed well, but exhibited more cultivar-specific variation, particularly in callus induction and shoot regeneration (Supplemental Table 2). These results indicate that increasing 2iP concentration improves consistency and may enhance genotype-independent regeneration success.

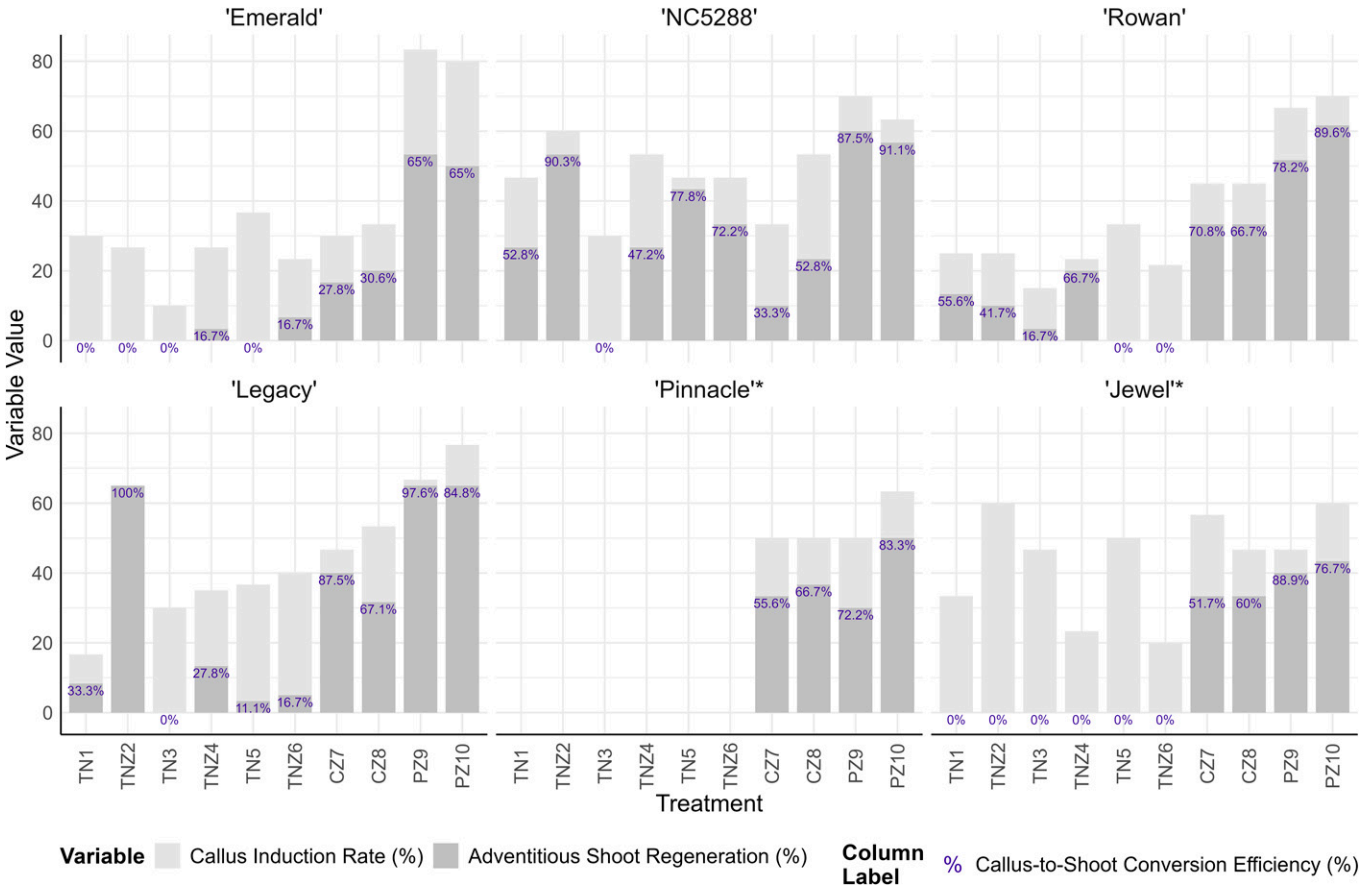

Fig. 5. Callus-to-shoot conversion efficiency in six *Vaccinium corymbosum* L. cultivars grown on woody plant medium–based regeneration media with treatments TN1 through PZ10. Light-gray bars indicate the callus induction rate, dark-gray bars indicate the adventitious shoot regeneration frequency, and purple text within bars denotes callus-to-shoot conversion efficiency, representing the percentage of callus that regenerated successfully into shoots. Data represent results after an 8-week culture period. * = observations made at the 4-week mark.

Conclusion

Our results confirm that ZT + 2iP (treatments PZ9 and PZ10) is the most effective cytokinin combination for promoting high-frequency callus induction, adventitious shoot regeneration, and callus-to-shoot conversion in highbush blueberry. TDZ-based treatments exhibited more variable and often ineffective responses, especially for recalcitrant cultivars such as Jewel and Pinnacle. ZT + CPPU treatments (CZ7 and CZ8) offer a promising middle ground, performing moderately across parameters, and are potentially useful for cultivars with intermediate responsiveness. 'NC5288' and 'Legacy' emerged as more adaptable genotypes capable of responding well to a broader range of PGR formulations. Most notably, treatment PZ10 demonstrated strong genotype-independent regeneration potential, offering a reliable option for standardizing tissue culture protocols in highbush blueberry. These findings provide valuable insights into optimizing in vitro propagation and may support future genetic transformation and breeding efforts.

Future work will focus on assessing the rooting efficiency of regenerated shoots, as high shoot regeneration alone does not ensure successful acclimatization to the soil. In many systems, high shoot yields are offset by poor rooting rates, limiting practical application. Rooting data will be essential to confirm the utility of each treatment for complete plant regeneration. In addition, treatment PZ10 is currently being tested on transformed blueberry plants to assess reproducibility in a genetic transformation context, with results to be published in future studies.

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