https://doi.org/10.17221/41/2026-JFS

Microclonal propagation of *Cotoneaster melanocarpus* as a tool for ecosystem restoration and resilience in Kazakhstan

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Citation: Aishuk Y., Sarsekova D. (2026): Microclonal propagation of *Cotoneaster melanocarpus* as a tool for ecosystem restoration and resilience in Kazakhstan. J. For. Sci., 72: 422–429.

Abstract: Degradation of steppe ecosystems in Kazakhstan requires propagation methods for native woody species. Seed propagation of *Cotoneaster melanocarpus* Fisch. ex Blytt is slow and irregular owing to dormancy. This study evaluated the effects of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) on *in vitro* shoot regeneration and multiplication. Overall, 200 apical and nodal explants from 40 visually healthy, naturally growing donor shrubs were cultured on Murashige and Skoog (MS) medium with five BAP/IBA treatments (T0–T4). Regeneration frequency, shoot number, shoot length, callus formation, and shoot-development patterns were summarised descriptively after 30 days. T3 produced the highest response (2.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} IBA): 37/40 explants regenerated (92.5%), yielding 5.2 shoots per explant and 6.3 cm mean shoot length. In T4 (3.0 mg·L^{-1} BAP and 1.0 mg·L^{-1} IBA), 24/40 explants regenerated (60.0%), with visible callus and apical abnormalities. Shoots in T1–T3 developed predominantly without visible intervening callus, although histology was not performed. Thus, T3 was the most favourable treatment tested for shoot multiplication. The findings are limited to shoot multiplication; rooting, acclimatisation, genetic fidelity, stress tolerance, and field performance require evaluation before restoration use.

Keywords: axillary bud proliferation; culture-medium optimisation; cytokinin–auxin balance; morphogenic response; Rosaceae

United Nations Sustainable Development Goal 15 prioritises restoration of degraded land, biodiversity conservation, and ecosystem resilience (United Nations 2015). This goal is key in arid and semi-arid regions, where drought, grazing, and woody-vegetation loss limit natural regeneration (Park et al. 2024). Kazakhstan has vast steppe, forest-steppe, semi-desert, and desert lands exposed to climate and human pressures (Rachkovskaya, Bragina 2012). Native plant material can restore cover, bind soil, and support biodiversity (Nef et al. 2021).

Native woody shrubs bind soil, form shelterbelts, enrich habitats, and aid ecosystem recovery. *Cotoneaster melanocarpus* Fisch. ex Blytt is a deciduous Rosaceae shrub found across Eurasia, including Kazakhstan (Dickoré, Kasperek 2010). *Cotoneaster* species have ornamental, melliferous, ecological, and medicinal value (Kicel 2020), while *C. melanocarpus* is valuable for conservation, shelterbelts, and dryland landscaping (Serafimovich et al. 2020). Wider practical use needs reliable propagation.

Cotoneaster seed propagation is limited by prolonged dormancy and slow, uneven germination.

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Dormancy may involve the seed coat and embryo physiology (Baskin, Baskin 2004). Seeds may need scarification followed by warm and cold stratification (Meyer 1988), yet emergence can vary by species, population, and seed lot (Bujarska-Borkowska, Suszka 2019; Marković et al. 2023). Even after such treatments, germination rates and timing may remain uneven, which limits the planned production of plant material. The main practical constraints are thus the length, complexity, and irregularity of germination, not the genetic variability of seedlings.

Sexual reproduction yields variation needed for adaptation and long-term population stability. The genetic makeup of source material affects the resilience of restored woody populations (Ivetić et al. 2016), but using one or a few clones may narrow their genetic base (Nef et al. 2021). Micropropagation should complement seed propagation and, in restoration programmes, include several genetically distinct, properly sourced donors. Clonal uniformity is useful within each donor line, but a restored population should mix lines from many donors, so that rapid multiplication does not reduce diversity. Overall, this balance is vital in long-term field restoration.

Plant tissue culture allows rapid multiplication of chosen genotypes under controlled conditions and also supports germplasm conservation and morphogenetic research (Long et al. 2022). However, woody-plant responses depend on genotype, explant source, and culture conditions (Kirillov et al. 2024). Shoot regeneration is strongly shaped by plant growth regulators. The cytokinin 6-benzylaminopurine (BAP) promotes axillary bud development, and cytokinin signalling interacts with auxin transport during bud activation (Wybouw, De Rybel 2019). Low indole-3-butyric acid (IBA) levels may alter cell division, shoot growth, and rooting competence. An unsuitable cytokinin–auxin balance may instead induce callus, compact shoots, poor elongation, hyperhydricity, or other abnormalities (Kaviani et al. 2023). Too little cytokinin may leave buds inactive, whereas high doses can impair shoot form and quality. Regulator combinations must thus be optimised for each species and explant type.

In vitro propagation has been reported for several *Cotoneaster* taxa, but responses vary among species and genotypes. Monier and Ochatt (1995) showed that multiplication and rooting in five genotypes depended on genotype and medium. BAP-containing media supported *C. wilsonii* micropropagation (Sivanesan et al. 2011), while indirect organogenesis was later obtained in the same species (Li et al. 2022). Serafimovich et al. (2020) proposed *in vitro* propagation for *C. melanocarpus* conservation.

Kirillov et al. (2023) developed a complete protocol for *C. melanocarpus* nodal explants using Murashige and Skoog or Quoirin–Lepoivre media with 0.2 mg·L^{-1} or 0.5 mg·L^{-1} 6-benzyladenine, 0.01 mg·L^{-1} IBA, and 0.5 mg·L^{-1} gibberellic acid. The highest response, 8.83 shoots per explant after 40 days, occurred on Murashige and Skoog (MS) medium containing 0.2 mg·L^{-1} 6-benzyladenine (BA), 0.01 mg·L^{-1} IBA, and 0.5 mg·L^{-1} GA_3. Rooting and acclimatisation were also achieved, yielding complete plants.

This study differs by using apical and nodal explants from wild donors, a wider BAP/IBA gradient without GA_3, and a 30-day period. It assessed regeneration frequency, shoot number and length, visible callus, and external apical abnormalities. Because BAP and IBA changed together, the study compared treatment combinations rather than their separate effects. Its aim was to describe shoot-regeneration and multiplication responses across five MS-based treatments and test whether intermediate combinations outperformed the hormone-free and highest-concentration treatments. The work covers only shoot multiplication; rooting, acclimatisation, genetic fidelity, stress tolerance, and field performance need further study. Future restoration should combine several genetically diverse donor lines rather than one clone.

MATERIAL AND METHODS

Plant material and culture establishment. Apical and nodal explants were collected in June 2025 from 40 naturally growing donor shrubs of *Cotoneaster melanocarpus* Fisch. ex Blytt in Kokshetau State National Nature Park, Kazakhstan. Donors were visually estimated to be 2–3 years old from their size and shoot development. All donor shrubs grew under natural field conditions within the sampled park. Morphologically typical shrubs were selected if they showed active current-season growth, normally developed foliage, and no visible disease, pest infestation, chlorosis, necrosis, mechanical damage, or severe stress. Young, non-flowering shoots were collected from well-illuminated parts of the shrubs. Drought tolerance, frost resistance, growth rate, and other adaptive traits were not tested, and no targeted selection for these traits was undertaken.

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Shoots were transported to the laboratory in sealed containers with moistened filter paper. Apical and nodal segments 1.0–1.5 cm long were excised as primary explants. They were washed under running tap water with mild detergent for 10–15 min, immersed in 70% (v/v) ethanol for 30 s, and treated with 1.2% sodium hypochlorite containing one drop of Tween-20 for 10 min. Explants were rinsed three times with sterile distilled water and transferred aseptically to culture media. The main procedural stages are summarised in Figure 1, which outlines collection, sterilisation, media transfer, and final assessment.

Culture media and experimental design. Murashige and Skoog basal medium (Murashige, Skoog 1962) containing 30 g·L^{-1} sucrose and 8 g·L^{-1} agar was used. The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Filter-sterilised growth-regulator stock solutions were added after the medium cooled to about 45 °C.

Five BAP/IBA treatments were evaluated (Table 1). Hormone-free MS medium was the control (T0), while T1–T4 contained increasing combined concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA). Because both regulators changed simultaneously, the experiment compared complete combinations and could not separate their individual effects.

Five comparable explants were prepared from each donor, and one was randomly assigned to each treatment, ensuring that all 40 donors were represented in each treatment. Apical and nodal explants were distributed as evenly as possible. Apical and nodal material was balanced across treatments without altering the donor-based allocation scheme. The 40 explants in each treatment were allocated to four separate culture vessels, with ten explants from ten different donors per vessel. Thus, the experiment comprised five treatments, four vessels per treatment, ten explants per vessel, and 200 explants. A vessel was the experimental unit; explants within it were subsamples.

Cultures were maintained at 23 ± 2 °C under a 16 h/8 h light/dark cycle and a photosynthetic photon flux density of 40–50 µmol·m^{-2}·s^{-1}. Responses were evaluated after 30 days in all five treatments.

Evaluation of regeneration and shoot development. An explant was considered regenerated when it produced at least one externally visible shoot. Regeneration frequency was calculated according to Equation (1):

$$\text{Regeneration frequency } (\%) = \frac{\text{number of regenerated explants}}{\text{total number of cultured explants}} \times 100 \quad (1)$$

For each treatment, regeneration was expressed as the number of regenerated explants out of 40 cultured explants (four replicates of ten explants each) and as the corresponding regeneration percentage.

Shoot development was assessed visually as occurring without externally visible intervening callus or in association with visible callus.

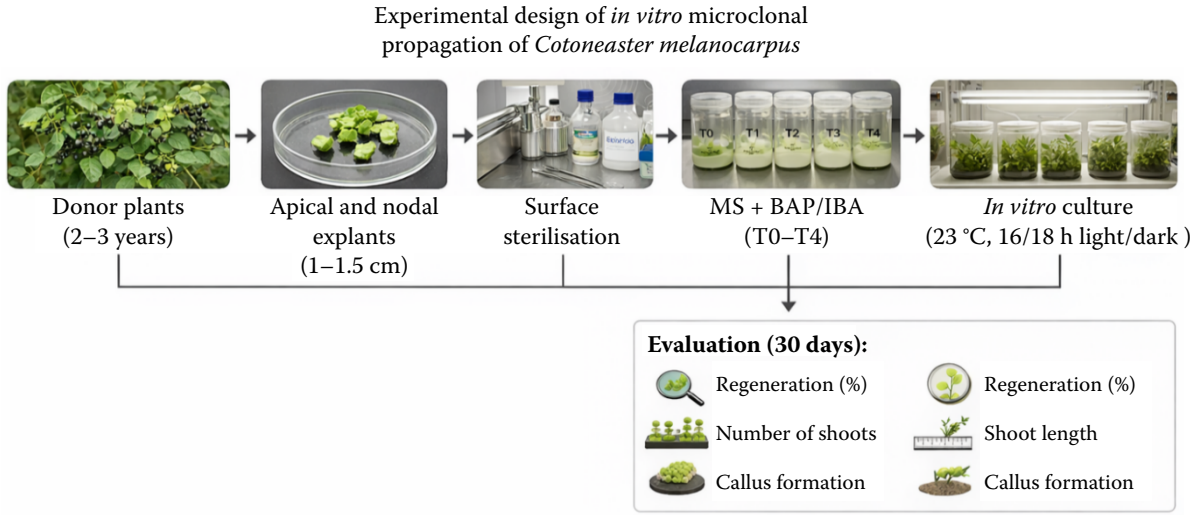

Figure 1. Main stages of the *in vitro* propagation procedure of *Cotoneaster melanocarpus*

MS – Murashige and Skoog medium; BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid

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Table 1. Experimental treatments and concentrations of BAP and IBA used for *in vitro* shoot multiplication of *Cotoneaster melanocarpus*

Treatment	BAP (mg·L^{-1})	IBA (mg·L^{-1})
T0	0.0	0.0
T1	0.5	0.1
T2	1.0	0.2
T3	2.0	0.5
T4	3.0	1.0

BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid

These categories were not treated as histological proof of direct or indirect organogenesis. Callus was scored visually as 0 (absent), 1 (very slight), 2 (slight), 3 (moderate), or 4 (abundant), and its location was recorded as the explant base, cut surface, or most of the explant surface. Apical regions were assessed for development, necrosis, deformation, uniformity, and colour intensity. No stereomicroscopic or histological examination was performed. Rooting, acclimatisation, genetic fidelity, stress tolerance, and field performance were not evaluated.

Statistical analysis. Available treatment-level results were summarised descriptively. Regeneration frequency was reported as *n*/40 and percentage; shoots per explant and shoot length were reported as recorded treatment-level means. Verified vessel-level observations needed to estimate within-treatment variation were unavailable; therefore, standard deviations, inferential tests, and multiple comparisons were not reported. Explants within a vessel were not treated as independent replicates. Differences among treatments are consequently interpreted as descriptive trends rather than statistically confirmed effects.

RESULTS

The descriptive responses of *Cotoneaster melanocarpus* explants after 30 days of *in vitro* culture are presented in Table 2. The evaluated parameters included the number and percentage of regenerated explants, the recorded mean number of shoots per explant, and mean shoot length.

The lowest recorded response occurred in the hormone-free control T0. Ten of the 40 explants regenerated, corresponding to a regeneration frequency of 25.0%. The recorded mean number of shoots was 0.8 per explant, and the mean shoot length was 1.2 cm. Weak shoot development and partial necrosis of apical tissues were observed in some control explants.

Among the treatments containing growth regulators, T3 produced the highest descriptive values. Thirty-seven of the 40 explants regenerated, corresponding to a regeneration frequency of 92.5%. The recorded mean number of shoots was 5.2 per explant, and the mean shoot length was 6.3 cm. In T2, 34 of the 40 explants regenerated (85.0%), with a recorded mean of 3.8 shoots per explant and a mean shoot length of 4.1 cm.

In T1, 27 of the 40 explants regenerated (67.5%), with a recorded mean of 2.6 shoots per explant and a mean shoot length of 2.8 cm. In T4, 24 of the 40 explants regenerated (60.0%); the recorded mean number of shoots was 2.1 per explant, and the mean shoot length was 3.0 cm. Thus, all three recorded parameters in T4 were numerically lower than those in T3. These numerical differences are presented descriptively and were not tested for statistical significance.

Visual characteristics of shoot development, callus formation and localisation, and the morphological condition of apical tissues are summarised in Table 3.

Table 2. Descriptive regeneration and shoot-development responses of *Cotoneaster melanocarpus* explants after 30 days on Murashige and Skoog (MS) media containing different combinations of BAP and IBA

Treatment	BAP (mg·L^{-1})	IBA (mg·L^{-1})	Regenerated explants, *n*/40 (%)	Shoots per explant	Shoot length (cm)
T0	0.0	0.0	10/40 (25.0)	0.8	1.2
T1	0.5	0.1	27/40 (67.5)	2.6	2.8
T2	1.0	0.2	34/40 (85.0)	3.8	4.1
T3	2.0	0.5	37/40 (92.5)	5.2	6.3
T4	3.0	1.0	24/40 (60.0)	2.1	3.0

BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid; values are descriptive treatment-level summaries; inferential statistical comparisons and multiple-comparison tests were not performed because verified culture-vessel-level observations were unavailable

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Table 3. Visual characteristics of shoot development, callus formation, and apex morphology in *Cotoneaster melanocarpus* explants after 30 days of *in vitro* culture

Treatment	BAP ($mg \cdot L^{-1}$)	IBA ($mg \cdot L^{-1}$)	Observed shoot-development pattern	Callus formation and localisation	Morphological condition of apices
T0	0.0	0.0	limited or absent shoot development	no visible callus	poorly developed; partial necrosis observed
T1	0.5	0.1	shoots developed without visible intervening callus	slight; localised at the explant base	small but visibly formed
T2	1.0	0.2	shoots developed predominantly without visible intervening callus	moderate; localised at the explant base	normally developed
T3	2.0	0.5	shoots developed without visible intervening callus	no visible callus	well-developed and visually uniform
T4	3.0	1.0	shoot development associated with visible callus	abundant; present over most of the explant surface	deformed and intensely green

Visual examination showed limited shoot development and partial necrosis in some T0 explants. In T1 and T2, visible shoots were accompanied by slight or moderate callus formation localised at the explant base. In T3, shoots developed without externally visible callus, and the apical regions appeared well-developed and visually uniform. In T4, abundant callus was observed over most of the explant surface, together with deformation and intense green colouration of the apical tissues. These observations were based on external morphology and were not interpreted as histological confirmation of direct or indirect organogenesis.

DISCUSSION

The response of *C. melanocarpus* differed from *C. horizontalis*, in which 3.0 $mg \cdot L^{-1}$ BA maximised shoot production and 2.0 $mg \cdot L^{-1}$ BA promoted elongation (Sayed, El-Kareim 2007). Here, T4 (3.0 $mg \cdot L^{-1}$ BAP and 1.0 $mg \cdot L^{-1}$) yielded lower values than T3, with 24 out of 40 explants regenerating (60.0%). The independent effects of BAP and IBA cannot be distinguished because both concentrations changed simultaneously. In *C. acuminatus*, woody plant medium with 0.5 $mg \cdot L^{-1}$ IBA produced up to 3.0 shoots per explant, whereas 0.1 $mg \cdot L^{-1}$ IBA supported rooting (Toma et al. 2014). The present study recorded 5.2 shoots per explant but did not evaluate rooting, indicating that multiplication and rooting require separate optimisation.

Similarly, 2.0 $mg \cdot L^{-1}$ BAP stimulated *Paulownia* shoot multiplication, but high concentrations reduced development (Salem et al. 2022). In apple, cytokinin signalling interacted with auxin transport during axillary bud outgrowth (Tan et al. 2019), providing a possible context for greater shoot production in BAP media. However, endogenous hormones and their transport were not measured, so the responses cannot be attributed exclusively to BAP.

Elevated cytokinin concentrations may reduce shoot quality. In *Salvia tomentosa*, increasing BA reduced normal shoot production and increased hyperhydricity (Martini et al. 2022). In *Hypericum aucheri*, 2.0 $mg \cdot L^{-1}$ BAP produced a comparable multiplication coefficient but caused chlorosis and basal callus (Cambaz, Çördük 2025). T4 likewise showed abundant visible callus and abnormal apical regions. Hyperhydricity, however, was not assessed anatomically or physiologically and cannot be confirmed.

T3 produced fewer shoots than *Rhus coriaria*, for which the same BAP and IBA combination yielded 12.3 shoots per explant and 100% rooting (Amiri, Mohammadi 2021). In *Bretschneidera sinensis*, lower BAP and auxin concentrations produced 90.37% regeneration, followed by rooting and molecular confirmation of genetic fidelity (Yan et al. 2024). Thus, *in vitro* responses vary by species and genotype, so a protocol for one woody species may fail in another.

Cross-study comparisons require caution, as media, explant types, hormone treatments, culture

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time, and response criteria differed. Numerical differences do not establish that one protocol is superior. They indicate that the *C. melanocarpus* response requires factorial testing of BAP and IBA, more donor genotypes, complete data for each vessel, and assessment of later stages under more than one controlled culture setting.

Shoot formation without a prolonged callus phase is preferable for clonal multiplication because callus-mediated regeneration may increase somaclonal variation. In *Haplophyllum tuberculatum*, BAP supported direct shoot regeneration, whereas callus-mediated regeneration was associated with detectable genetic variation (Alsafran et al. 2022). Shoots in T1–T3 developed predominantly without externally visible intervening callus, but external morphology was not confirmed histologically or genetically and does not prove direct organogenesis. A complete *Quillaja saponaria* protocol included multiplication, 92.85% rooting, and above 84% acclimatisation survival (Guerra et al. 2025), whereas this study evaluated only shoot regeneration and multiplication.

Restoration-oriented micropropagation should use multiple genetically distinct donor clones. Population genetic assessment is recommended when selecting source populations and donor material (Van Rossum et al. 2022). In threatened plants, representation of several genetic families can preserve diversity and support early establishment (Schäfer et al. 2020). Mixing regional sources may increase diversity, although their proportional representation can change during propagation and establishment (St. Clair et al. 2020). A global meta-analysis found that restored woody populations often contain less genetic diversity than natural references (Wei et al. 2023). Mass multiplication of one genotype should therefore be avoided.

Limitations include sampling one geographical area, phenotypic rather than genetic selection of 40 donors, simultaneous changes in BAP and IBA, and unavailable verified vessel-level data for inferential analysis. Shoot development patterns lacked histological and molecular confirmation. Rooting, acclimatisation, genetic fidelity, stress tolerance, and field performance were not evaluated. Conclusions are therefore limited to descriptive *in vitro* shoot regeneration and multiplication responses under the five tested BAP/IBA treatments.

CONCLUSION

Among the five treatments evaluated, MS medium supplemented with 2.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} IBA produced the highest descriptive response during the *in vitro* regeneration and multiplication of *Cotoneaster melanocarpus* shoots. In this treatment, 37 of 40 explants regenerated (92.5%), with recorded means of 5.2 shoots per explant and a shoot length of 6.3 cm after 30 days. Shoot development occurred without externally visible intervening callus, and the apical regions appeared well-developed and visually uniform. However, the regeneration pathway was not confirmed histologically or genetically.

The findings are limited to the shoot-regeneration and multiplication stage and should not be interpreted as statistically confirmed treatment effects because verified culture-vessel-level observations were unavailable. Root induction, acclimatisation, genetic fidelity, stress tolerance, and field performance require further investigation before a complete micropropagation protocol can be proposed. Any future restoration application should use material derived from multiple genetically characterised donor clones rather than from a single selected genotype.

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Received: May 18, 2026
Accepted: August 10, 2026
Published online: August 17, 2026