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# *In vitro* Micropropagation of *Berberis chitria* (Lindl.) - A Rare Medicinal Plant from Himachal Pradesh, India

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*Key words: Berberis chitria* (Lindl.), *In vitro* micropropagation, Callus induction, Shoot induction, Root induction.

## Abstract

An efficient *in-vitro* micropropagation protocol has been developed for *Berberis chitria* (Lindl.) - a rare medicinal plant from Himachal Pradesh, India using seeds and *in-vitro* nodal explants. The effects of different concentrations of BAP and $GA_3$ for *in-vitro* seed germination was investigated and consequently 6.0 mg/l BAP + 0.0 mg/l $GA_3$ was reported to be excellent for seed germination. Callus induction from leaf explants from aseptically grown seedling were also studied by using various concentrations of BAP and 2, 4-D and showing 94.26 ± 0.12% response at the concentration of 2.0 mg/l BAP + 0.4 mg/l 2, 4-D. The highest percentage of multiple shoot induction was obtained (100.00 ± 0.01% with an average of 4.50 ± 0.3 shoots) on MS medium augmented with 6.0 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l IAA. Whereas, the combined concentration of 1.0 mg/l NAA + 1.0 mg/l IBA + 0.5 mg/l IAA showed highest (76.05 ± 0.09%) root induction with an average of 3.84 ± 0.27 roots per shoot. The survival rate of these plantlets under greenhouse condition was 85%. This research communication provides first record about the *in-vitro* multiplication of *B. chitria*, collected from Himachal Pradesh Himalayas of India.

## Introduction

*Berberis* (L.) is as genus which comprises high medicinal values. It belongs to the family Berberidaceae and also called tree turmeric and Indian berberry. These well-developed shrubs are commonly scattered in temperate and sub-tropical regions of the world (Chauhan 1999). Berberidaceae family reported with 12 genera and 600 species (Keshtkaran et al. 2022). One of the major genus i.e. *Berberis* of the family Berberidaceae comprises around 500 species native to Asia, Europe, Africa and America (Samant and Pant 2003, Bargali et al. 2022). There were total 55 species of *Berberis* reported from India.

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Whereas, 21 species have already been reported from Himachal Pradesh, India (Chowdhery and Wadhwa 1984; Rao et al. 1998). Numerous medicinal properties have already been reported for the various species of the genus such as: antiemetic, antimicrobial, antioxidant, antipyretic, antiinflammatory, hypotensive, sedative and cholagogue actions (Ghareeb et al. 2013, Gidik 2021). These medicinal properties are due to numerous phytoconstituents present in different parts of these plants. The roots of this plant contain most important phyto-constituents such as; berberine, palmatine, taxalamine, barbamine, oxyberberine, aromoline, protoberbemine, etc. So, keeping in view, it is more significant to study the various species within the genus *Berberis.* In the present research work, a new and efficient protocol for micropropagation has been developed for *B. chitria* (Lindl.) species. It is well known fact that berberine is utilized as a medicine in many diverse forms and its content in *B. chitria* species is also noticed with higher amount particularly in roots and stem parts, therefore the *B. chitria* species is mostly used as a adulterant or substitute to *B. aristata* (Srivastava et al. 2006). To reduce the burden on one of the most exploited species i.e. *B. aristata*, it is required to increase the biomass production of *B. chitria* by mean of micropropagation technique to meet the need of the pharmaceutical industries. As it is well reported that, the seeds of *Berberis* species possess embryonic dormancy but still plants are being mostly propagated by seeds and stem cuttings (Dirr and Heuser 1987, Swingle 1939). To reduce the exploitation of related species, and to enhance the biomass production the *in-vitro* propagation technique for *B. chitria* was commenced to develop a well-organized protocol for future use. Except only one report *viz.*, Pandey et al. (2013), not much related research literature is available till date particularly for *B. chitria* species from Himalayan region, further the research data on *in-vitro* micropropagation is also not reported from Himachal Pradesh Himalayas. Therefore, the present study might be a significant step towards the development of micropropagation technique and conservation of one of the most important, rare medicinal herb of Himachal Pradesh for further future utilization so as to decrease the burden on other most exploited species of the genus *Berberis*.

## Materials and Methods

For *in-vitro* studies the plant materials were collected from wild region of Sirmour District of Himachal Pradesh, India and then maintained in experimental farms of Herbal-cum-Botanical Garden of the University. Further, for *in-vitro* micropropagation of *Berberis chitria* (Lindl.), seeds from cultivated accessions were collected during the months of June-July, 2021 from wild sources and analysed in laboratory of Department of Botany. Plants specimens were identified by the faculty of Department of Botany, Eternal University, Baru Sahib, Himachal Pradesh (India) and submitted to NBPGR Centre Shimla (H.P.).

Collected seeds of species were meticulously washed for 30 min under the running tap water. Further these seeds were treated with 10% (v/v) Teepol (Himedia, Ltd. India)

for 15 min and followed the continuous washing for next 10 min under running tap water. Seeds were then washed thrice with double distilled water and further sterilization was done under aseptic conditions in a Laminar Airflow Chamber made of Popular Traders, India. $HgCl_2$ 0.1% (w/v) was used for surface sterilization for 7.0min and thoroughly washed with autoclaved double distilled water several times to remove traces of mercuric chloride ($HgCl_2$) before inoculation. For better percentage of germination the sterilized seeds were cultured in different concentrations and combinations of BAP (2.0, 4.0, 6.0 mg/l) and $GA_3$ (2.0, 4.0, 6.0 mg/l).

The shoot tips from *in-vitro* developed nodal segments were cut into appropriate size and cultured on MS basal medium which contained 3% (w/v) sucrose. Prior to autoclaving the pH of the medium was adjusted to 5.8 and with addition of 0.8% (w/v) agar into it. The medium was then dispensed in 15 ml aliquots into culture tubes (25 x 150 mm) and 50 ml in glass jam jars (400 ml) and capped with non-absorbent cotton plugs. The entire medium was then autoclaved at 1.1 kg/$cm^2$ pressure and 121$^o$C temperature for 15 min. A fixed photoperiod was maintained by cool white florescent tubes (Philips Ltd. India) for 16h at 3000 lux light intensity in the culture room along with 50-70 % relative humidity and 25 ± 2$^o$C temperature. The leaves were collected from *in-vitro* germinated seedlings and cut into small pieces (~0.4$cm^2$). Then transferred to MS medium supplemented with different concentrations and combinations of BAP (1.0, 2.0, 3.0 mg/l) and 2, 4-D (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mg/l). The same conditions of the growth chamber were maintained as stated earlier. After seven weeks of incubation of cultures, the callus induction responses such as responsive percentage, texture, color and size were recorded.

MS basal medium supplemented with various concentrations of plant growth regulators was used for shoot proliferation, therefore different concentrations of benzylaminopurine (BAP) 3.0, 6.0, 9.0 mg/l combined with various concentrations of Kn (Kinetin) (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 1.0, 2.0, 3.0 mg/l) and IAA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 1.0, 2.0, 3.0 mg/l) were used for culture initiation and multiplication of shoots from *in-vitro* developed nodal segments. All cultures were transferred to the fresh medium after 2-3 weeks. The mean number of shoots, length and percentage of culture response were evaluated after 4 weeks of inoculation.

*In vitro* raised shoots (3-7 cm) were separated and transferred to full strength MS basal medium containing 3% (w/v) sucrose solidified with 0.8% (w/v) agar. The medium was further enriched with various concentrations of growth hormones *viz.,* NAA ($\alpha$-Naphthalene acetic acid) 1.0, 2.0, 3.0 mg/l and IBA (Indole 3-butyric acid) (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 1.0, 2.0, 3.0 mg/l) and IAA (Indole Acetic acid) (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 1.0, 2.0, 3.0 mg/l). After 4 weeks of inoculation, the number of roots/shoot, length of roots and percentage of root induction were also noted. Plantlets with well-developed roots were removed carefully from the culture vessels, washed gently under running tap water to remove the remains of agar. Then transferred to the pots containing decontaminated mixture of soil, sand and farmyard manure in the ratio of 1:1:1. These plantlets were kept

under transparent polythene membranes/sheets for 2 weeks to confirm high humidity and then kept in open diffused light for hardening. The survived plants were then transferred to pots containing garden soil after 15-20 days and maintained in the greenhouse for acclimation.

## Results and Discussion

For *in vitro* seed germination the effects of various BAP and $GA_3$ concentrations was investigated. The concentration of 6.0 mg/l BAP + 0.0 mg/l $GA_3$ was found to be excellent for germination i.e. 65.46 ± 0.13% (Table 1 and Fig. 1a). Callus induction from leaf explants from aseptically germinated seedling was also studied by using various concentrations of BAP and 2, 4-D and exhibiting 94.26 ± 0.12% response at a concentration of 2.0 mg/l BAP + 0.4 mg/l 2, 4-D (Table 2).

**Table 1. Effects of different ABA and $GA_3$ concentration on *in vitro* seed germination of *Berberis chitria* (Lindl.) (n = 3).**

| Sl. No. | BAP (mg/l) | $GA_3$ (mg/l) | Germination (%) |
|---|---|---|---|
| Control | 0.0 | 0.0 | 0.00 ± 0.00 |
| 1. | 2.0 | 0.0 | 15.24 ± 0.12 |
| 2. | 4.0 | 0.0 | 25.36 ± 0.36 |
| 3. | 6.0 | 0.0 | 65.46 ± 0.13 |
| 4. | 0.0 | 2.0 | 28.00 ± 0.18 |
| 5. | 0.0 | 4.0 | 22.05 ± 0.05 |
| 6. | 0.0 | 6.0 | 47.12 ± 0.04 |
| 7. | 2.0 | 2.0 | 38.35 ± 0.10 |
| 8. | 4.0 | 4.0 | 34.12 ± 0.04 |
| 9. | 6.0 | 6.0 | 29.54 ± 0.20 |

The results exhibited the best response in terms of multiple shoot production, when these nodal explants cultured on MS medium supplemented with various combined concentrations of BAP, Kn and IAA. The early signs of bud initiation and bud breaking were first observed within 14 days and followed by production of multiple shoots within 20-35 days when nodal explants cultured on MS medium. As per the results, the highest percentage of multiple shoot induction was 100.00 ± 0.01% showing average 4.50 ± 0.3 number of shoots on the medium when augmented with 6.0 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l IAA. It is well known fact that from three cytokinins, BAP always considered to be superior to other two i.e. Kn and IAA, but combined results are more prominent than use of single growth hormone, in terms of better shoot development and number of shoots per node, followed by 89.52 ± 0.42% shoot induction and an average of 3.12 ± 0.11

numbers of shoots in the medium supplemented with 3.0 mg/l BAP + 2.0 mg/l Kn + 2.0 mg/l IAA. The individual concentration i.e. 6.0 mg/l BAP showed 42.62 ± 0.08% of shoot induction with an average of 2.13 ± 0.03 numbers of shoots (Table 3). Thus, from the present results, it found that BAP showed more effectiveness and superior for shoot induction if used in combination with Kn and IAA (Figs. 1b-c). Previously, it was well reported that for shoot induction, BAP independently acts as a better agent for production (Chirangini et al. 2005; Karthikeyan et al. 2007; Sharma et al. 2014). One of the previous report, Arena et al. (2000) observed that due to oxidation and production of phenolic compounds browning of the shoots was also observed which finally lead to the death of the shoots. This is in contrast to the findings of Hara et al. (1994) for *Berberis* species.

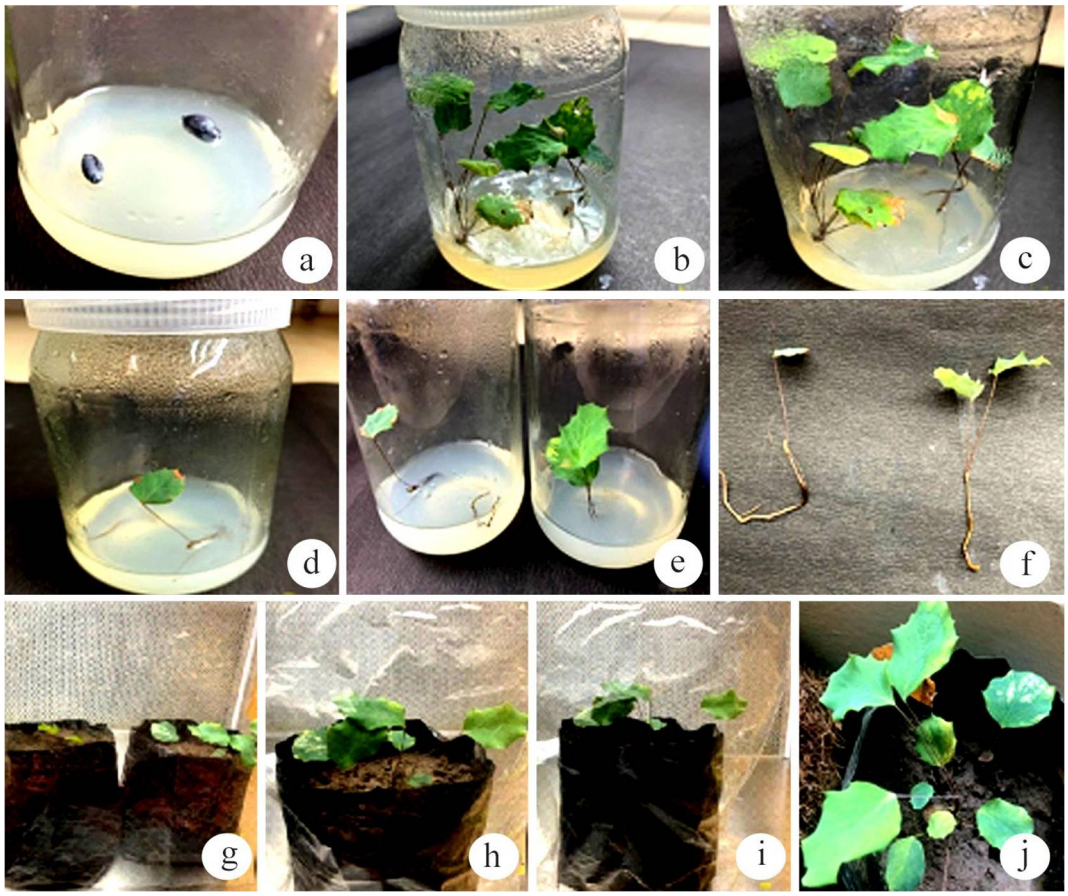
**Table 2. Effects of BAP and 2, 4-D on callus induction from leaf explants of aseptically germinated seedling of *Berberis chitria* (Lindl.) (n = 3).**

| Sl. No. | BAP (mg/l) | 2, 4-D (mg/l) | Responsive explants (%) | Texture | Color | Growth |
|---|---|---|---|---|---|---|
| Control | 0.0 | 0.0 | 0.00 ± 0.00 | NA | NA | NA |
| 1. | 1.0 | 0.2 | 68.25 ± 0.12 | Friable | Light yellow | Good |
| 2. | 2.0 | 0.4 | 94.26 ± 0.12 | Friable | Green | Excellent |
| 3. | 3.0 | 0.6 | 45.36 ± 0.26 | Friable | Light yellow | Good |
| 4. | 1.0 | 0.8 | 42.28 ± 0.27 | Compact | Green | Poor |
| 5. | 2.0 | 1.0 | 35.24 ± 0.21 | Friable | Light Yellow | Good |
| 6. | 3.0 | 1.2 | 26.28 ± 0.20 | Compact | Brown | Poor |

For root initiation, the well-developed shoots were isolated and cultured on MS medium having different concentrations of NAA, IBA and IAA. The frequency and number of roots per shoot varied with individual and combined concentrations of these growth hormones. Among all the concentrations, the combined concentration of 1.0 mg/l NAA + 0.5 mg/l IBA + 0.5 mg/l IAA showed highest 76.05 ± 0.09% root induction with an average 3.84 ± 0.27 number of roots per shoot (Figs 1d-f), followed by 67.50 ± 0.49% root induction with an average 2.29 ± 0.46 number of roots per shoot in the medium containing 1.0 mg/l NAA + 1.0 mg/l IBA + 0.0 mg/l IAA (Table 4). Therefore, it is clear that the combined concentration of various growth hormones *viz*., NAA, IBA and IAA is an perfect treatment for root induction. As per previous reports, IBA was the most suitable auxin for rooting in many plant species (Shahzad et al. 2007; Parveen and Shahzad 2010).

After 4 weeks, well rooted plantlets were obtained and their morphometric characters were studied. These plantlets were removed from the culture vessels, washed gently under running tap water and planted in small pots (plastic pots) containing a

potting mixture of sand, soil and farmyard in the ratio of 1 : 1 : 1 (Figs 1g-j). The potted plantlets were covered by transparent polythene sheet to maintain suitable humidity. After proper acclimation, the survival rate of these plantlets under greenhouse condition was 85%. It has been already reported that the nodal explants are the best source for multiple shoot induction in many medicinal plants (Vadawale et al. 2006).




Figs. 1(a-j). *In-vitro* propagation of *B. chitria* Lindl (a) Initiation of *in-vitro* seed germination on media after 10-15 days of inoculation. (b) Well grown seedling development on medium after 25-35 days of culture. (c) Shoot multiplication after 85-90 days. (d-e) Well elongated roots in root induction media. (f) *In-vitro* developed roots (g-i) Acclimatization of the raised plants inside culture room condition for 25-30 days with plastic covering. (j) Hardening of *in-vitro* well growing plants in plastic pot.

A number of medicinal plants were also cultured for conservation and multiplication by using various tissue culture techniques (Hasan et al. 2008; Vijayakumar et al. 2010; Sridhar et al. 2011). Numerous pharmacological activities and clinical studies have already been reported from different *Berberis* species which show their importance as a medicinal plant. Berberine and berbamine alkaloids present in *Berberis* species have anti-

oxidant, anti-inflammatory, hepatoprotective, hypotensive and anti-hyperglycemic properties as reported by various research groups (Koncic et al. 2010b; Singh and Kakkar 2009; Semwal et al. 2009; Tiwari and Khosa 2010; Soffar et al. 2001). Extracts prepared by boiling the stem bark and root of *B. aristata*, *B. chitria*, *B. lycium* in water and used since the ancient times to cure for bleeding piles, skin diseases, ulcers jaundice, inflamed liver (Rajasekaran and Kumar 2009). The above protocol can be exploited for commercial propagation and conservation of valuable medicinal plants belongs to the genus *Berberis*.

**Table 3. Effects of BAP, Kn and IAA on shoot multiplication from the *in vitro* developed nodal segment of *Berberis chitria* (Lindl.) on basal MS medium (*n=3*).**

| Sl. No. | BAP (mg/l) | Kn (mg/l) | IAA (mg/l) | Mean no. of shoots/node | Mean length of shoots (cm) | Culture response (%) |
|---|---|---|---|---|---|---|
| Control | 0.0 | 0.0 | 0.0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 1. | 3.0 | 0.0 | 0.0 | 1.36 ± 0.02 | 1.35 ± 0.02 | 26.35 ± 0.49 |
| 2. | 6.0 | 0.0 | 0.0 | 2.13 ± 0.03 | 2.36 ± 0.10 | 42.62 ± 0.08 |
| 3. | 9.0 | 0.0 | 0.0 | 2.13 ± 0.04 | 2.85 ± 0.28 | 35.25 ± 0.21 |
| 4. | 3.0 | 1.0 | 0.0 | 2.56 ± 0.07 | 3.46 ± 0.20 | 28.24 ± 0.13 |
| 5. | 6.0 | 2.0 | 0.0 | 2.48 ± 0.11 | 1.45 ± 0.09 | 34.65 ± 0.06 |
| 6. | 9.0 | 3.0 | 0.0 | 2.79 ± 0.14 | 2.75 ± 0.09 | 55.84 ± 0.34 |
| 7. | 3.0 | 0.0 | 1.0 | 1.65 ± 0.11 | 0.59 ± 0.09 | 38.25 ± 0.48 |
| 8. | 6.0 | 0.0 | 2.0 | 2.98 ± 0.51 | 2.57 ± 0.10 | 46.36 ± 0.03 |
| 9. | 9.0 | 0.0 | 3.0 | 1.75 ± 0.56 | 2.15 ± 0.14 | 52.45 ± 0.20 |
| 10. | 3.0 | 0.5 | 0.5 | 2.65 ± 0.11 | 2.00 ± 0.01 | 58.46 ± 0.27 |
| 11. | 6.0 | 1.0 | 1.0 | 4.50 ± 0.31 | 4.15 ± 0.09 | 100.00 ± 0.01 |
| 12. | 9.0 | 1.5 | 1.5 | 2.85 ± 0.40 | 2.85 ± 0.16 | 78.56 ± 0.07 |
| 13. | 3.0 | 2.0 | 2.0 | 3.12 ± 0.11 | 3.05 ± 0.11 | 89.52 ± 0.42 |
| 14. | 6.0 | 2.5 | 2.5 | 1.38 ± 0.08 | 1.40 ± 0.16 | 81.00 ± 0.90 |
| 15. | 9.0 | 3.0 | 3.0 | 1.37 ± 0.19 | 2.70 ± 0.06 | 65.85 ± 0.05 |

In the present research report, an improved and efficient protocol has been developed which optimized the combinations for better, superior shoot and root induction in *B. chitria* (Lindl.). The nodal explant culture technique and micropropagation reported here offers an effective method of multiplication and conservation of this important medicinal plant species for future use. Simultaneously, the same protocol can also be applied for *in-vitro* micropropagation and large scale biomass production of various other species of the genus *Berberis* and also for conservation of some other medicinal plants taxa.

**Table 4. Effects of NAA, IBA and IAA on rooting of *in-vitro* raised shoots of *Berberis chitria* (Lindl.) on the basal MS medium (n = 3).**

| Sl. No. | NAA (mg/l) | IBA (mg/l) | IAA (mg/l) | Mean no. of roots/shoot | Mean length of roots (cm) | % of root induction |
|---|---|---|---|---|---|---|
| Control | 0.0 | 0.0 | 0.0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 1. | 1.0 | 0.0 | 0.0 | 1.25 ± 0.09 | 2.1 ± 0.10 | 16.25 ± 0.26 |
| 2. | 2.0 | 0.0 | 0.0 | 1.38 ± 0.08 | 1.36 ± 0.04 | 19.57 ± 0.05 |
| 3. | 3.0 | 0.0 | 0.0 | 1.50 ± 0.03 | 1.52 ± 0.10 | 36.85 ± 0.49 |
| 4. | 1.0 | 1.0 | 0.0 | 2.29 ± 0.46 | 2.54 ± 0.07 | 67.50 ± 0.49 |
| 5. | 2.0 | 2.0 | 0.0 | 0.56 ± 0.08 | 1.85 ± 0.09 | 45.65 ± 0.21 |
| 6. | 3.0 | 3.0 | 0.0 | 1.28 ± 0.04 | 0.35 ± 0.06 | 32.50 ± 0.11 |
| 7. | 1.0 | 0.0 | 1.0 | 2.77 ± 0.15 | 2.80 ± 0.15 | 65.25 ± 0.18 |
| 8. | 2.0 | 0.0 | 2.0 | 2.54 ± 0.08 | 1.95 ± 0.03 | 56.20 ± 0.27 |
| 9. | 3.0 | 0.0 | 3.0 | 1.39 ± 0.07 | 2.75 ± 0.14 | 48.35 ± 0.05 |
| 10. | 1.0 | 0.5 | 0.5 | 3.84 ± 0.27 | 3.15 ± 0.16 | 76.05 ± 0.09 |
| 11. | 2.0 | 1.0 | 1.0 | 1.05 ± 0.10 | 1.02 ± 0.17 | 36.57 ± 0.14 |
| 12. | 3.0 | 1.5 | 1.5 | 1.39 ± 0.12 | 1.32 ± 0.07 | 27.25 ± 0.37 |
| 13. | 1.0 | 2.0 | 2.0 | 0.79 ± 0.14 | 1.45 ± 0.07 | 19.23 ± 0.19 |
| 14. | 2.0 | 2.5 | 2.5 | 1.46 ± 0.09 | 1.87 ± 0.02 | 42.13 ± 0.05 |
| 15. | 3.0 | 3.0 | 3.0 | 1.10 ± 0.02 | 1.95 ± 0.04 | 22.26 ± 0.13 |

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