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ORIGINAL ARTICLE

Novel approaches to improve rooting of microshoots, acclimatization and plant growth of Pyrodwarf pear rootstock

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Abstract

Ex vitro root formation of micro cuttings of Pyrodwarf pear rootstock (*Pyrus communis* L.) using floating perlite bed and the effects of Indole-3-butric acid (IBA) treatments on the growth performance after five months were determined. Micro cuttings were dipped in 25, 50, 100 or 150 mg L^{-1} IBA solutions for 10 s for *ex vitro* rooting, except for the control. The floating perlite bed cultures were kept for 4 weeks at 25 ± 2ºC with a photoperiod of 16 h light (35 µmol•m^{-2}•s^{-1}). At the end of the rooting experiment, there were no statistically significant differences among treatments in terms of rooting percentages (77.8%-81.1%), number of roots (1.9–2.5) and the length of the longest root (22.8–36.8 mm). In the plant growth experiment, *ex vitro* rooted micro cuttings treated with 25 and 50 mg L^{-1} IBA and the control were maintained under greenhouse conditions for 5 months to determine the growth performance of the plants. Growth performance was higher in terms of stem diameter, root and stem dry weights of plants rooted with 50 mg L^{-1} IBA determined as 4.5 mm, 196.5 mg and 704.7 mg, respectively. In conclusion, it was found that the floating perlite bed method was effective in *ex vitro* rooting and acclimatization. Ex vitro rooting with 50 mg L^{-1} IBA improved plant growth performance in greenhouse.

Key message

Ex vitro rooting and acclimatization of Pyrodwarf micro cuttings were achieved successfully using floating perlite bed. Micro cuttings quick-dipped in 50 mg L^{-1} IBA exhibited improved growth performance.

Keywords *Pyrus* · Micro cutting · Ex vitro · Floating perlite bed · IBA

Abbreviations

IBA	Indole- 3- butyric acid
BAP	6-Benzylaminopurine
GA_3	Gibberellic acid
ARs	Adventitious roots
MS	Murashige and Skoog

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Introduction

Pear cultivars (*Pyrus* spp.) are mostly propagated by budding or grafting on rootstocks belonging to different *Pyrus* spp. and *Cydonia oblonga* (quince). The choice of an appropriate rootstock for a cultivar is based on many characteristics such as graft compatibility, response to biotic and abiotic stress factors, growth vigour, precocity, yield and fruit quality, tree anchorage and suckering (Bell et al. 1996). Especially since the twentieth century, breeding programs have been carried out in many parts of the world to develop new clonal rootstocks for modern pear cultivation. Pyrodwarf® is a dwarfing clonal rootstock (*P. communis*) developed through a breeding program initiated in the 1980s at the Geisenheim Research Institute in Germany (Jacob 1998). This rootstock has no incompatibility problems with pear cultivars (*P. communis*) and is tolerant to calcareous soils and low winter temperatures (Jacob 1998; Federico et al. 2009; Sharifi et al. 2018). Mass production of this rootstock, which can also be propagated by cuttings,

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is only possible by micropropagation (Necas and Kosina 2008; Kaviani et al. 2023).

In plants, roots formed from non-root tissues, such as cuttings, are adventitious roots (ARs). This distinguishes adventitious roots from primary and lateral roots. Unlike lateral roots, adventitious roots may be initiated from a variety of cell types and tissues, not just pericycle cells. Phytohormones regulate every step of AR formation and development (Lakehal and Bellini 2019). Auxins, especially indole-3-butyric acid (IBA), are a group of hormones that induce AR formation in cuttings. IBA stimulates root primordia formation, increases the number of ARs and upregulates the expression of genes (MdWOX11, MdLBD16 and MdLBD29) involved in AR formation (Bai et al. 2020).

In micropropagation, micro shoots proliferated by in vitro subcultures can be rooted in vitro or *ex vitro*. The next stages are acclimatization and development of the plants. These last stages, together with rooting are critical phases of micropropagation that directly affect the success of micropropagation and can lead to significant economic losses during the propagation of rootstocks (Hartmann et al. 2011; Soumare et al. 2021).

Currently, in vitro rooting is widely used in micropropagation and the acclimatization of plantlets removed from in vitro conditions is carried out over a period of 2–4 weeks under greenhouses or tunnels equipped with mist or fogging systems and gradually lowering the relative humidity. However, many losses in plants can occur in this way (Thakur et al. 2008; Chandra et al. 2010; Hartmann et al. 2011; Soumare et al. 2021). On the other hand, the *ex vitro* rooting method, in which the acclimatization of the plantlets occurs during root formation reduces the production time and cost in micropropagation. Moreover, the *ex vitro* rooted plantlets develop a better root and shoot system influencing their survival rate compared to in vitro rooting (Yeo and Reed 1995; Aygun and Dumanoglu 2015; Oakes et al. 2016; Ibrahim Ahmed Osman and Dumanoglu 2023). Nevertheless, the widespread *ex vitro* rooting method requires growing units such as relative humidity-controlled greenhouses and plant growth chambers, etc. to prevent moisture loss in micro cuttings at the beginning. These units increase the cost of micropropagation and sometimes irregularities, especially in the relative humidity, can greatly reduce the success of rooting and plant production. On the other hand, floating perlite bed is reported to be a much easier, low cost and effective method for acclimatization together with *ex vitro* rooting of micro cuttings (Clapa et al. 2013a). In this method, the planting medium is perlite bed laid on tap water in a plastic tub. Perlite is moistened by absorbing water underneath, increasing the relative humidity of the surrounding environment of micro cuttings. In addition perlite can also retain air, which helps with aeration that is important for root survival and growth. In these cultures, the use of fertilizers or biostimulators is not necessary and there is no need for mechanical or automatic adjustment of relative humidity around the plant materials (Clapa et al. 2013a). To date, micro shoots have been successfully *ex vitro* rooted using this technique in *Rubus fruticosus* x *R. idaeus*, *Amelanchier canadensis*, *R. fruticosus, R. idaeus, Vaccinium macrocarpon, V. corymbosum, Rosa hybrida* (Clapa et al. 2013a, 2015; Fira et al. 2014), *Prunus cerasus x P. canescens* (Gisela 5) (Clapa et al. 2013b), *Paulownia* spp. (Clapa et al. 2014), *Malus domestica* (Fira et al. 2015; Clapa et al. 2016) and *Syringa vulgaris* (Orsolya et al. 2017).

In studies on micropropagation of Pyrodwarf rootstock, it has been reported that micro cuttings were in vitro rooted under aseptic conditions and acclimatization was carried out in humidity-controlled conditions such as greenhouse and plant growth chambers (De Paoli et al. 2002; Ruzic et al. 2011; Vujovic et al. 2012; Nourmohammadi et al. 2015; Kandi et al. 2018; Sharifi et al. 2018; Kaviani et al. 2023). *Ex vitro* rooting of Pyrodwarf micro cuttings has not been reported before. Furthermore, the floating perlite bed technique has not been previously investigated in *Pyrus* species.

In this study, *ex vitro* rooting of micro cuttings treated with or without IBA using the floating perlite bed was investigated and subsequent plant growth performance of the *ex vitro* rooted micro cuttings were determined in greenhouse conditions.

Materials and methods

Micro cutting preparation

Micro shoots obtained from in vitro subcultures of Pyrodwarf pear clone rootstock (*P. communis* L.) were used for the preparation of micro cuttings. MS basal medium (Murashige and Skoog 1962) with 1/3 ammonium nitrate supplemented with 1.0 mg L^{-1} 6-Benzylaminopurine (BAP), 0.3 mg L^{-1} gibberellic acid (GA_3) and 3% sucrose was used for shoot proliferation in subcultures. The pH of the medium was adjusted to 5.8 and solidified with 0.7% agar. The medium was distributed 50 mL into 250 mL Erlenmeyer flasks and autoclaved for 20 min at 121 ºC under a pressure of 106 kPa (1 atm). Eight micro shoots were planted on fresh medium in each flask, and subcultures were repeated at 4-week intervals. Cultures were incubated in a climate room at 25 ± 2 ºC with a 16-h light (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 8-h dark photoperiod. After the third subculture, the agar residues at the bottom of the proliferated shoot explants were washed and micro cuttings with apex and two leaves about 2 cm in length were prepared.

Ex vitro rooting of micro cuttings

In this study, *ex vitro* rooting of micro cuttings was carried out using floating perlite bed (Clapa et al. 2013a). The floating perlite bed was prepared by slowly laying 3 L of agricultural perlite (3–6 mm in diameter) evenly on the surface of 3 L of tap water filled in a square plastic tub 25 cm width, 11 cm height and 6 L in volume. The outside of the tub was covered with black plastic sheeting to prevent light transmission (Fig. 1).

Micro cuttings were treated with 25, 50, 100, 150 mg L^{-1} IBA prepared by dissolving IBA powder (Sigma 15386) in 96% ethyl alcohol and reached to the final volume with deionized distilled water. The micro cuttings were placed upright in small glass containers with a diameter and height of 2.5 cm each containing 50 micro cuttings for each treatment. A volume of 5 mL of IBA solution was added into the small glass containers carrying micro cuttings. In this way, the bottom 0.5 cm of the micro cuttings was exposed to IBA for 10 s by quick-dip method. The micro cuttings were immediately removed from the glass containers and planted to the floating perlite bed for *ex vitro* rooting.

The floating perlite cultures were incubated for 4 weeks in a climate room at 25 ± 2ºC with a 16 h light (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$), 8 h dark photoperiod. At the end of the incubation period, rooting and survival percentage, number of roots, length of the longest root, rooting level which is the classification of rooting performance (1 = poor, 2 = moderate, 3 = good, 4 = very good), callus diameter, fresh and dry root weights were determined. Plantlet height, fresh and dry shoot weights were also determined. Dry weight was assessed by drying roots and shoots in an oven at 65 °C for 3 days until they reached constant weight. The experiment was repeated twice.

Plant growth experiment in the greenhouse

In the second experiment of this study, the effects of low concentrations of IBA used for *ex vitro* rooting of micro cuttings, on the growth of plants in the greenhouse were investigated. Control, 25 mg L^{-1} and 50 mg L^{-1} IBA concentration, which were found effective in the rooting of micro cuttings in the first experiment, were used for the second experiment. Micro cuttings, initially dipped into 25 or 50 mg L^{-1} IBA solution for 10 s or not (control), were incubated in the climate room for 4 weeks using the floating perlite bed. The *ex vitro* rooted and at the same time acclimatized plants were then planted in 100 mL plastic pots containing a 3:1 mixture of peat and perlite (v/v) and moved to the greenhouse. The pots were covered with a shade net from 1 m above for 1 week in order to prevent high light intensity in the greenhouse. At the end of 5 months, plant height and stem diameter 10 cm above the root collar were measured, fresh and dry weights of roots and stem without foliage were determined.

Experimental design and statistical analysis

The experiments were designed according to the randomized plot design with three replicates. In *ex vitro* rooting of micro cuttings, five treatments (control, 25, 50, 100 and 150 mg L^{-1} IBA) x three replicates × 15 micro cuttings = 225 micro cuttings were used. In the plant growth experiment in the greenhouse, three treatments (control, 25 and 50 mg L^{-1} IBA) x three replicates × 20 plants = 180 plants were used. The data were subjected to analyses of variance (ANOVA) by the Minitab Package Program (MINITAB Inc., trial version). Significant differences were determined by Duncan's test ($P \leq 0.05$). Percentage data were statistically analyzed after arcsine transformation.

Results

Ex vitro rooting of micro cuttings

Ex vitro rooting of micro cuttings using the floating perlite bed, revealed that there was no significant difference between the control and IBA treatments in terms of rooting percentage (%), root number, longest root length and plantlet height. As an average of all treatments, around 80% of the micro cuttings rooted successfully and all of the plantlets



(a) (b) (c)

Fig. 1 Floating perlite bed for *ex vitro* rooting. **a**, **b** Preparation of the perlite bed. **c** Pyrodwarf micro cuttings planted in the floating perlite bed

survived. However, significant differences were found in terms of rooting level assessed using a scale of 1–4, root and shoot fresh and dry weights among treatments. The root fresh and dry weights per micro cuting were significantly higher in the control compared to IBA treatments. The rooting level was also the highest in the control followed by 50 and 150 mg L^{-1} IBA. While the highest shoot fresh weight was in micro cuttings treated with 50 and 100 mg L^{-1} IBA, the highest shoot dry weight was recorded in the control, 50 and 100 mg L^{-1} IBA followed by 25 and 150 mg L^{-1} IBA (Table 1, Fig. 2).

Plant growth performance

The differences in terms of growth parameters such as stem diameter, root and stem fresh and dry weights were found statistically significant among control and IBA treatments. Plant height however was not statistically different among treatments (Table 2).

Although the initial assessment of growth parameters of *ex vitro* rooted micro cuttings revealed a better performance in terms of growth parameters in the control compared to the IBA concentrations (Table 1), the growth parameters of the 5-month-old plants exhibited lower values in the control (Table 2). This finding suggested that IBA treatments had shown significant effects on growth parameters, the significantly highest values were recorded in the 50 mg L^{-1} IBA, followed by 25 mg L^{-1} IBA concentrations (Table 2, Fig. 3).

Stem diameter was greater by 25%, root fresh weight by 142%, root dry weight by 170%, stem fresh weight by 77% and stem dry weight by 82% in 5-month-old plants treated with 50 mg L^{-1} IBA compared to the control. The increase in the percentages of the above mentioned parameters were 6%, 79%, 73%, 21% and 23%, respectively in 5-month-old plants treated with 25 mg L^{-1} IBA compared to the control.

Table 1 Effect of indole-3-butyric acid (IBA) on *ex vitro* rooting of micro cuttings of Pyrodwarf rootstock after 4 weeks in floating perlite bed in growth room*

Parameter	Control	IBA concentration (mg L^{-1})				*P*
		25	50	100	150	
Rooting (%)	80.0 ± 2.4	78.7 ± 4.4	77.8 ± 2.2	81.1 ± 4.0	80.0 ± 6.0	**0.925**^{ns}
Root number per micro cuttings	2.3 ± 0.2	1.9 ± 0.2	2.5 ± 0.2	2.3 ± 0.2	1.9 ± 0.2	**0.152**^{ns}
Longest root length (mm)	36.8 ± 0.2	23.6 ± 0.2	28.4 ± 0.2	22.8 ± 0.2	25.9 ± 0.2	**0.080**^{ns}
Rooting level (1–4)	2.0 ± 0.1 **a**^{b}	1.2 ± 0.2 **b**	1.7 ± 0.1 **ab**	1.4 ± 0.2 **b**	1.5 ± 0.2 **ab**	**0.047***
Root fresh weight (mg)	11.5 ± 1.9 **a**	5.5 ± 0.7 **b**	7.8 ± 1.0 **b**	6.0 ± 1.2 **b**	7.4 ± 1.1 **b**	**0.019***
Root dry weight (mg)	1.3 ± 0.3 **a**	0.5 ± 0.1 **b**	0.8 ± 0.1 **b**	0.6 ± 0.1 **b**	0.8 ± 0.1 **b**	**0.024***
Plantlet height (mm)	20.8 ± 0.5	19.2 ± 1.3	21.6 ± 0.8	22.3 ± 1.1	22.0 ± 1.4	**0.323**^{ns}
Shoot fresh weight (mg)	77.1 ± 3.7 **b**	68.8 ± 3.7 **b**	94.3 ± 7.3 **a**	85.1 ± 6.0 **ab**	68.9 ± 6.4 **b**	**0.019***
Shoot dry weight (mg)	19.0 ± 1.0 **a**	14.5 ± 1.0 **b**	19.2 ± 1.0 **a**	16.4 ± 1.1 **ab**	13.6 ± 1.0 **b**	**0.002****

*IBA applied to the micro cuttings for 10 s by quick-dip method. Rooting level, 1 = poor, 2 = medium, 3 = good, 4 = very good. Different letters in the same row indicate the statistical difference between treatmens according to Duncan test ($P \leq 0.05$). *, ** $P \leq 0.05$ and $P \leq 0.01$ statistically significant, ^{ns} statistically not significant

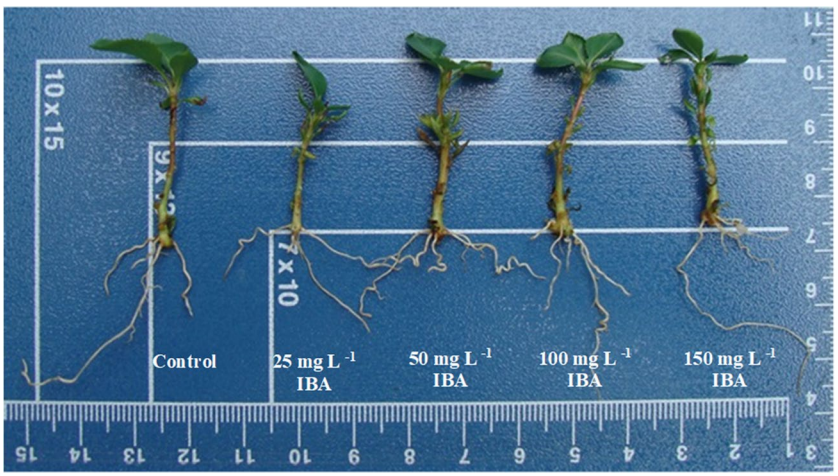


Fig. 2 *Ex vitro* rooted micro cuttings (plantlets) of Pyrodwarf using the floating perlite bed initially treated with 25, 50, 100, 150 mg L^{-1} indole-3-butyric acid (IBA) and the control

Table 2 Plant growth performance of Pyrodwarf plantlets rooted with or without indole-3-butyric acid (IBA) using the floating perlite bed ex vitro

Growth Parameter	IBA concentrations (mg L^{-1})*			*P*
	Control	25	50	
Plant height (mm)	118.2 ± 1.3	111.7 ± 6.6	118.7 ± 4.1	**0.515**^{ns}
Stem diameter (mm)	3.6 ± 0.1 **b**	3.8 ± 0.1 **b**	4.5 ± 0.1 **a**	**0.001*****
Root fresh weight (mg)	483.9 ± 72.4 **b**	868.0 ± 134.0 **ab**	1173.0 ± 126.0 **a**	**0.015***
Root dry weight (mg)	72.8 ± 7.1 **c**	126.3 ± 20.0 **b**	196.5 ± 12.8 **a**	**0.003****
Stem fresh weight (mg)	876.2 ± 63.8 **b**	1063.7 ± 80.6 **b**	1547.8 ± 30.6 **a**	**0.001*****
Stem dry weight (mg)	387.4 ± 17.7 **c**	477.8 ± 35.4 **b**	704.7 ± 16.7 **a**	**0.000*****

*IBA was used for *ex vitro* rooting. Different letters in the same row indicate the statistical difference between treatmens according to Duncan test ($P \leq 0.05$). ^{ns} statistically not significant. *, **, *** $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ statistically significant, respectively

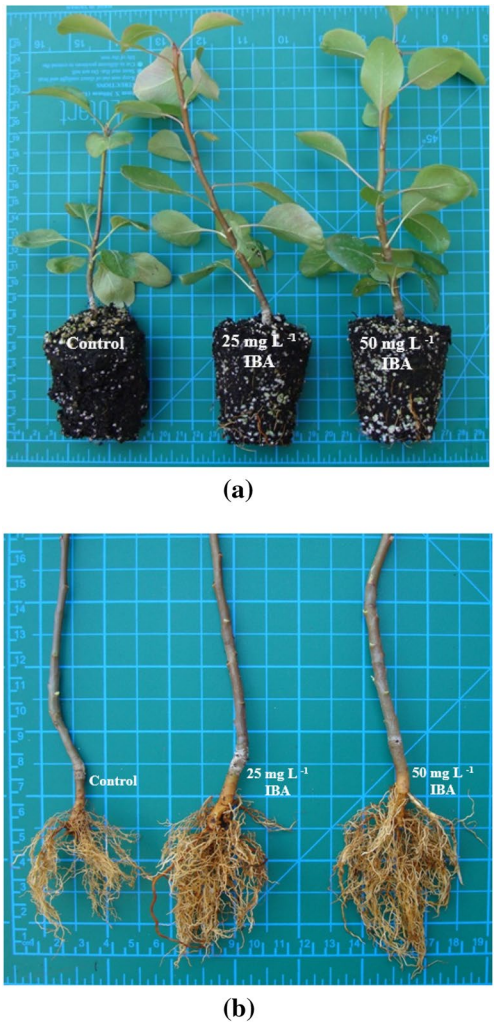


Fig. 3 **a** Growth performance and **b** root development of *ex vitro* rooted 5-months' old Pyrodwarf rootstock

Discussion

Plant production on Pyrodwarf rootstock, which is an important dwarfing clonal pear rootstock for intensive pear orchards, is usually carried out by micropropagation method (Kaviani et al. 2023). Micropropagation of Pyrodwarf rootstock is currently performed by in vitro rooting and subsequent acclimatization in humidity-controlled conditions (Ruzic et al. 2011; Vujovic et al. 2012; Kandi et al. 2018; Kaviani et al. 2023). However, *ex vitro* rooting that produces plants with healthy lateral roots and root hairs similar to those in the natural environment has not been studied in Pyrodwarf micro cuttings. *Ex vitro* rooted plantlets may have a much higher chance of survival in the orchard than in vitro rooted plantlets (Yan et al. 2010; Pessanha et al. 2022). In addition, rooting and acclimatization of the plantlets occur at the same time during *ex vitro* rooting, which saves resources, energy and time, and can reduce production costs by around 70% (Ranaweera et al. 2013). Floating perlite bed is reported to be a much easier, low cost and effective method for acclimatization together with *ex vitro* rooting of micro cuttings (Clapa et al. 2013a). There are no previous studies using the floating perlite bed in pears. For the first time this method was used in our research for *ex vitro* rooting of Pyrodwarf pear rootstock. With this technique, high percentage of acclimatized Pyrodwarf plantlets with healthy root and shoot development was obtained within just 4 weeks, ready to be treansferred to the greenhouse.

IBA is a widely used rooting hormone in the auxin family, that promotes the formation and development of root tip meristem and adventitious roots in plants (Frick and Strader 2018). IBA has been shown to promote in vitro rooting of Pyrodwarf rootstock (Ruzic et al. 2011; Vujovic et al. 2012; Nourmohammadi et al. 2015). Our findings revealed that the difference between the control and IBA treatments was not statistically significant in terms of rooting percentage, root number and longest root length during *ex vitro* rooting of the Pyrodwarf rootstock using floating perlite bed. This finding suggests

that the use of auxin is not necessarily required for *ex vitro* rooting of micro cuttings using floating perlite bed. Moreover, the highest fresh and dry root weights were recorded in the control (Table 1). Kaviani et al. (2023) reported that in vitro rooting could not be achieved in auxin-free media (control) in Pyrodwarf. However, similar to our findings, it was reported that auxin was not required for *ex vitro* rooting using floating perlite bed method in *Paulownia* species (Clapa et al. 2014), Royal Gala apple cultivar (Fira et al. 2015) and some apple rootstocks (Clapa et al. 2016). Different from these studies, we extended the experiment for a period of 5 months in order to determine the growth performance of *ex vitro* rooted plantlets under greenhouse conditions. Interestingly, it was found that the use of auxin (25 and 50 mg L^{-1} IBA) during *ex vitro* rooting had a significant effect in improving plant growth parameters under greenhouse conditions. This notable finding was attributed to the critical role of auxins in cell division and cell expansion (Frick and Strader 2018) and in the improvement of root quality that influences the absorption of nutrients from the growing medium indirectly leading to improved shoot development (Shiri et al. 2019).

Conclusion

The findings of this study strongly recommend *ex vitro* rooting of micro cuttings using floating perlite bed as a rapid, successful and low cost method for micropropagation of Pyrodwarf rootstock. This method not only allows rooting in a short period of time also allows the acclimatization of the plantlets at the same time. Furthermore, treatment of micro cuttings with IBA solution before placing in floating perlite bed for *ex vitro* rooting is reccommended to improve subsequent plant growth and development.

Author contributions FN performed investigation and formal analysis; HD performed conceptualization, methodology, supervision, writing the original draft; GS performed data curation, writing, review, editing and visualization. All authors have read and approved the final document.

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Data availability All data supporting the findings of this study are available within the paper.

Declarations

Competing interests The authors declare that there is no conflict of interest.

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