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Standardizing ovule age for *in ovulo* embryo rescue in seedless grape (*Vitis vinifera*) breeding under the subtropical region

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ABSTRACT

The present study was carried out during 2018–22 at ICAR-Indian Agricultural Research Institute, New Delhi to identify the appropriate ovule age (days after pollination-DAP) to rescue the *in ovulo* embryo after pollination in various seedless grape (*Vitis vinifera* L.) genotypes and their cross combinations. The research revealed that in *in vitro* ovule culture establishment, ovule maturity, and *in ovulo* embryo germination increased significantly from 18–33 DAP ovule age and declined thereafter from 33–43 DAP. The highest ovule culture establishment (85.17%), ovule maturity (71.38%), and *in ovulo* embryo germination (25.54%) and ovule growth (2.02 mm^2) were consistently observed at 33 DAP ovule age in most of the grape genotypes and cross combinations. The time required for embryo germination was notably reduced at 33 DAP (105.24 days). The optimal ovule age for embryo rescue varied among different grape genotypes. It was noticed that 23 DAP ovule age is ideal for Centennial Seedless; 28 DAP for cross combination, Beauty Seedless × Pusa Urvashi; and 33 DAP for genotypes like Perlette, Beauty Seedless, Pusa Urvashi, Pusa Trishar, Pusa Aditi, Flame Seedless; and cross combination like Pusa Aditi × Beauty Seedless, Pearl of Csaba × Beauty Seedless, Pusa Urvashi × Perlette and Pusa Trishar × Perlette; and 38 DAP for Pusa Swarnika for maximum *in ovulo* embryo rescue and germination recovery under sub-tropics.

Keywords: Germination, Hybridization, *In ovulo* embryo rescue, Ovule age, Seedless grape

Grape (*Vitis vinifera* L.) is a highly valuable remunerative fruit crop, largely known for its immense nutraceutical value. Despite being originated in the Mediterranean temperate climate, it is widely cultivated in tropical and subtropical regions of the world (Possingham 2003). India ranked 7th in global annual grape production of 3.4 million metric tonnes from 0.16 million hectares of area (MoAFW 2021). The grapes are cultivated worldwide for table consumption, winemaking, raisins, and juice production, with seedless grapes being particularly valued for their use as table grapes and in raisin production (Li *et al.* 2014, Akkurt *et al.* 2019). Many cultivated seedless grape varieties are stenospermocarpic, where fertilization occurs, but embryo and/or endosperm development ceases shortly after fertilization, leading to seed abortion at various growth stages (Giancaspro *et al.* 2022). In the subtropical regions, where the grape-growing season is very short, the development of early seedless grape genotype is the major objective for the crop improvement programme, apart from high fruit quality, better nutritional value, processing attributes, and resistance to biotic and abiotic stresses (Somkuwar and Gawande 2022).

Traditional hybridization is an important technique used to obtain new seedless cultivars. However, this method is often yielding a low proportion (less than 15%) of seedless progeny in the F_1 generation, furthermore, it is also highly time-consuming (Jiao *et al.* 2018, Atak 2023, Moniruzzaman *et al.* 2023). However, seedless × seeded and seedless × seedless crosses face challenges due to stenospermocarpic nature, which leads to hybrid embryo abortion at an early stage of the berry development of the grape. Further, the standardization of ideal ovule age is very different from temperate or tropical regions since the berry maturity is very short. To overcome these challenges and shorten the breeding process, *in ovulo* embryo rescue is the most promising technique, involving the excision and culture of embryos before they abort (Li *et al.* 2020). The developmental progression through *in vitro* culture of an ovule to a normal seedling is influenced by several factors, including parental genotype, culture medium composition, and embryo age (Li *et al.* 2018, Jiao *et al.* 2018, Li *et al.*

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2020). Therefore, standardizing the embryo age for different grape genotypes and cross combinations is of utmost importance for seedless grape breeding in the sub-tropical region. The objective of our study was to identify the optimal embryo age for rescuing them after pollination (sampling time) in various seedless grape parental genotypes and new hybrid combinations, to expedite grape improvement breeding programmes.

MATERIALS AND METHODS

The present study was carried out during 2018–22 at ICAR-Indian Agricultural Research Institute, New Delhi. About 8–11-year-old grape genotypes, viz. Beauty Seedless (BS), Flame Seedless (FS), Centennial Seedless (CS), Perlette (PER), Pusa Urvashi (PU), Pusa Trishar (PT), Pusa Aditi (PA), Pusa Swarnika (PSW), and Pearl of Csaba (PoC) maintained in the experimental vineyard at ICAR-Indian Agricultural Research Institute, New Delhi were selected for hybridization. A total of 23 cross combinations were attempted involving early maturing seeded × seedless and seedless × seedless cross combinations, thereafter five responsive cross combinations including, PU × BS, PU × PER, PT × PER, PA × BS, and POC × BS were selected for further studies. The standard cultural practices were followed for fertilization, irrigation, and pest control during the investigation.

The hybridization process involved tagging and bagging of healthy inflorescences at the swollen bud stage, followed by emasculation and hand pollination from 7.00–11:00 AM was carried out. After 7 days, bags were removed, and developing berries rescued from controlled pollination and open-pollinated (OP) berries of different parental genotypes were collected for ovule extraction at 5-day intervals from 18–43 days after pollination (DAP).

The collected berries underwent a thorough cleaning with 0.1% liquid soap solution (Tween-20) for 15 min followed by agitation in tap water and surface sterilization with 10% sodium hypochlorite (NaOCl) for 15–20 min followed by 3–4 washing with sterile double-distilled water under laminar air-flow. Ovules were then carefully extracted and cultured in the Erlenmeyer flask containing an established medium. Matured embryos were subjected to chilling treatment (at 4°C for 45 days in the dark) and scarification before being transferred for *in vitro* germination. Cultures are maintained in the culture room under cool-white fluorescent illumination (40 µM m-2 s-1) with 25 ± 2°C temperature and photoperiod of 16/8 h light and dark cycle. The data on the following parameters, viz. berries for ovule excision%, culture establishment%, ovule maturity%, ovule growth (mm^2), *in ovulo* embryo germination per cent, and days to embryo germination were recorded every 15-day intervals.

The percent data were subjected to Arcsine transformation values for the statistical analyses. Analysis of variance (ANOVA) was performed according to completely randomized design (CRD) using the R software version 4.2.1 and mean comparisons were performed with the least significance test (LSD).

RESULTS AND DISCUSSION

Effect of ovule age on in vitro in ovulo *embryo culture establishment*: To accelerate breeding programmes in India, many studies have been performed to investigate the optimal developmental stage to conduct embryo rescue in grapes (Midani 2001, Singh *et al.* 2011), subtropical peaches (Srivastav *et al.* 2003) and citrus fruits (Singh *et al.* 2020). However, there are very few studies have been attempted on *in-ovulo* embryo rescue in grapes in subtropical India. To simplify the grape embryo rescue procedure, our current work employed an *in ovulo* embryo rescue culture method.

In this study, we investigated the impact of ovule age on the establishment of *in vitro* embryo cultures across various grape genotypes and cross combinations (Fig. 1), following a 30-day inoculation period on culture media. Notably, the

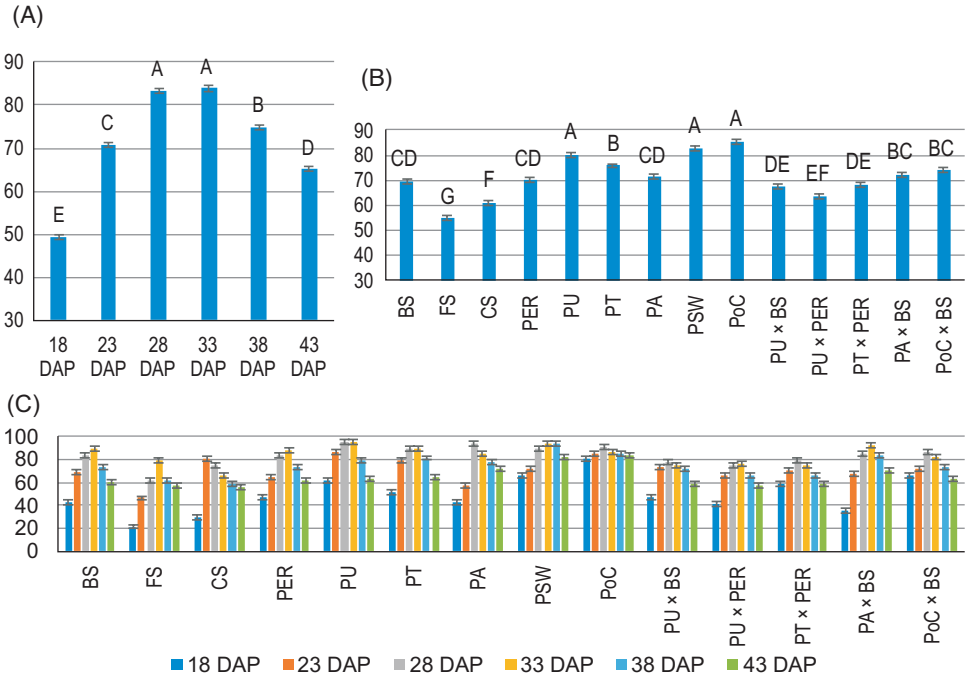

Fig. 1 Effect of ovule age on *in vitro in ovulo* embryo culture establishment percentage after 30 days of inoculation (DAI) in various grape genotypes and their cross combinations.
(A) Ovule age; (B) Genotype; (C) Ovule age and genotype interaction. DAP, Days after pollination.
Treatment details are given under Materials and Methods.

stage of ovule growth, independent of genotype, exhibited a pronounced influence on *in vitro* culture establishment. The highest percentage of culture establishment (83.86%) was observed at 33 DAP, while the lowest percentage (49.45%) was recorded at 18 DAP. Many researchers reported that the genotype-specific differences in ovule response to culture conditions emphasize the significant role of genetic factors in determining the success of *in vitro* culture establishment (Li *et al.* 2015, Singh *et al.* 2020, Giancaspro *et al.* 2022).

In line with the previous studies, we observed the impact of grape genotype on culture establishment was evident (Fig. 1). Genotype PoC exhibited the highest culture establishment (85.52%), while genotype FS had the lowest (54.85%) culture establishment, irrespective of ovule age. Among the cross combinations, PoC × BS showed the highest culture establishment (73.94%), which was statistically similar to 72.32% in cross PA × BS, whereas PU × PER had the lowest culture establishment (63.75%). Additionally, the two-way interaction indicated a significant overall increase in ovule culture establishment percentage with advancing ovule age up to 33 DAP, followed by a declining trend from 33 DAP to 43 DAP (Fig. 1). Genotype PU displayed the highest culture establishment (95.66%) at 28 DAP, while FS had the lowest (21.67%) at 18 DAP. In cross combinations, the highest establishment (91.90%) was observed in PA × BS at 33 DAP, and the lowest (35.56%) was observed at 18 DAP ovule age, aligning with previous research (Kanamadi *et al.* 1999, Li *et al.* 2015, Benke *et al.* 2021), highlighting the influence of ovule age on *in vitro* survival.

Table 1 Effect of ovule age on *in vitro* ovule maturity after 60 days of inoculation in various grape genotypes and their cross combinations

Genotype/Cross combination	Ovule age (days after pollination)						
	18	23	28	33	38	43	Mean
BS (OP)	0.00	42.24	67.34	78.22	67.51	65.40	53.45^{D}
	(0.90)	(40.56)	(55.17)	(62.21)	(55.28)	(54.00)	(47.00)
FS (OP)	0.00	37.9	34.56	51.86	45.14	40.95	35.07^{G}
	(0.90)	(38.02)	(36.03)	(46.09)	(42.23)	(39.81)	(36.33)
CS (OP)	0.00	67.50	54.44	63.65	57.27	53.94	49.47^{DE}
	(0.90)	(55.27)	(47.57)	(52.95)	(49.20)	(47.28)	(44.72)
Per (OP)	20.90	29.95	36.67	42.66	37.93	31.72	33.30^{FG}
	(27.22)	(33.2)	(37.29)	(40.80)	(38.04)	(34.30)	(35.26)
PU (OP)	22.79	65.56	76.92	83.65	75.87	67.46	65.38^{B}
	(28.53)	(54.09)	(61.32)	(66.18)	(60.61)	(55.25)	(53.98)
PT (OP)	34.44	70.46	73.22	85.68	77.22	63.79	67.47^{B}
	(35.96)	(57.10)	(58.87)	(67.8)	(61.52)	(53.03)	(55.25)
PA (OP)	26.67	33.67	66.06	76.08	67.31	58.18	54.66^{C}
	(31.11)	(35.49)	(54.39)	(60.75)	(55.16)	(49.73)	(47.70)
PSW (OP)	38.33	75.63	83.62	87.78	89.63	76.60	75.27^{A}
	(38.27)	(60.45)	(66.16)	(69.57)	(71.25)	(61.10)	(60.21)
PoC (OP)	53.06	46.46	71.78	78.28	76.94	73.47	66.66^{B}
	(46.78)	(42.99)	(57.94)	(62.25)	(61.33)	(59.03)	(54.76)
PU × BS	27.77	50.56	77.12	66.25	62.63	62.63	57.83^{C}
	(31.82)	(45.34)	(61.45)	(54.51)	(52.34)	(52.34)	(49.53)
PU × PER	21.62	35.37	53.89	55.56	47.36	41.81	42.60^{E}
	(27.72)	(36.51)	(47.25)	(48.21)	(43.51)	(40.3)	(40.77)
PT × PER	18.04	32.78	42.33	50.00	39.26	37.41	36.64^{F}
	(25.15)	(34.94)	(40.61)	(45.02)	(38.82)	(37.73)	(37.27)
PA × BS	44.81	65.56	78.30	83.65	71.59	58.89	67.13^{B}
	(42.05)	(54.09)	(62.27)	(66.18)	(57.82)	(50.15)	(55.05)
PoC × BS	39.29	65.70	75.53	81.33	69.28	54.91	64.34^{B}
	(38.83)	(54.18)	(60.38)	(64.43)	(56.37)	(47.84)	(53.36)
Mean	31.61^{E}	51.38^{D}	63.70^{B}	70.33^{A}	63.21^{B}	56.23^{C}	
	(34.23)	(45.81)	(52.98)	(57.03)	(52.69)	(48.60)	
Factor	DAP	Genotype (G)	G × DAP				
LSD ($P \leq 0.05$)	1.663	2.540	6.222				

*Values in parentheses indicate Arcsine transformed data. The values with different alphabetical letters differ significantly at 5%. The capital letters show differences among the mean value for both factors.

Treatment details are given under Materials and Methods.

Effect of ovule age on in vitro *ovule maturity*: Recent studies suggest that the duration of ovule culture plays a crucial role in recovering viable hybrids from seedless genotype crosses. Extending embryo growth during ovule culture can enhance embryo rescue efficiency, as more advanced embryos have a higher chance of survival and subsequent plantlet development (Liu *et al.* 2008). The results of the present study clearly showed that ovule age had a significant impact on ovule maturity after 60 days of inoculation (Table 1). The highest percentage (70.33%) of ovule maturity was observed at 33 DAP, while it was lowest (31.61%) at 18 DAP. Among the various genotypes studied, PSW exhibited the highest maturity percentage (71.34%), while PER had the lowest (31.61%). Regarding cross combinations, PA × BS had the highest maturity (67.13%), and PT × PER had the lowest (36.64%) ovule maturity. When the interaction between ovule age and genotypes was studied, it was found that genotype PSW registered the significantly highest ovule maturity (89.63%) at 38 and 33 DAP ovule age respectively. In cross combinations, cross PA × BS (83.65%) registered the highest ovule maturity. Conversely, the lowest ovule maturity percentage (18.04%) was recorded in the cross-combination PT × PER at 18 DAP. Genotypes BS, FS, and CS did not reach maturity at 18 DAP ovule age due to necrosis of ovules. These findings are consistent with previous studies, highlighting the significance of understanding embryonic development stages, where early-stage embryos face lower prospects of maturing into plants (Midani 2002, Li *et al.* 2013, Li *et al.* 2015, Wang *et al.* 2016, Giancaspro *et al.* 2022).

Effect of ovule age on in vitro *ovule growth (mm²) during incubation and maturation*: The stage of embryo rescue is a crucial factor in the recovery of zygotic seedlings, and it depends on the stage at which embryo abortion occurs (Singh *et al.* 2020). We examined the influence of ovule age on *in vitro* ovule growth during a 60-day incubation period for various grape genotypes and cross combinations (Fig. 2).

Ovule growth was determined by subtracting the initial value from the final value. We observed that *in ovulo* embryo growth ranged from 1.61–2.02 mm² with the increase of ovule age from 18–33 DAP, followed decline trend from 33–43 DAP, irrespective of genotype. When considering the mean ovule growth values across different genotypes and crosses, irrespective of ovule age and incubation period, the highest growth (2.22 mm²), was noted in the genotype PSW. However, the lowest ovule growth value (1.39 mm²) was observed in genotype PER. However, the increase in growth was at its lowest (1.80 mm²) at 24-days ovule age. This may be attributed to the lag phase of berry development when ovule growth in vivo slows down (Kennedy 2002).

Effect of ovule age on in vitro in-ovulo *embryo germination*: The germination capacity of hybrid embryos can be affected by the embryo's genetic potential and stage of development (Villoria *et al.* 2005, Guo *et al.* 2013). It is evident that parental genetics and the timing of ovule sampling play pivotal roles in determining success (Li *et al.* 2015, Giancaspro *et al.* 2022). We investigated the influence of ovule age on *in vitro in ovulo* embryo germination (Fig. 3).

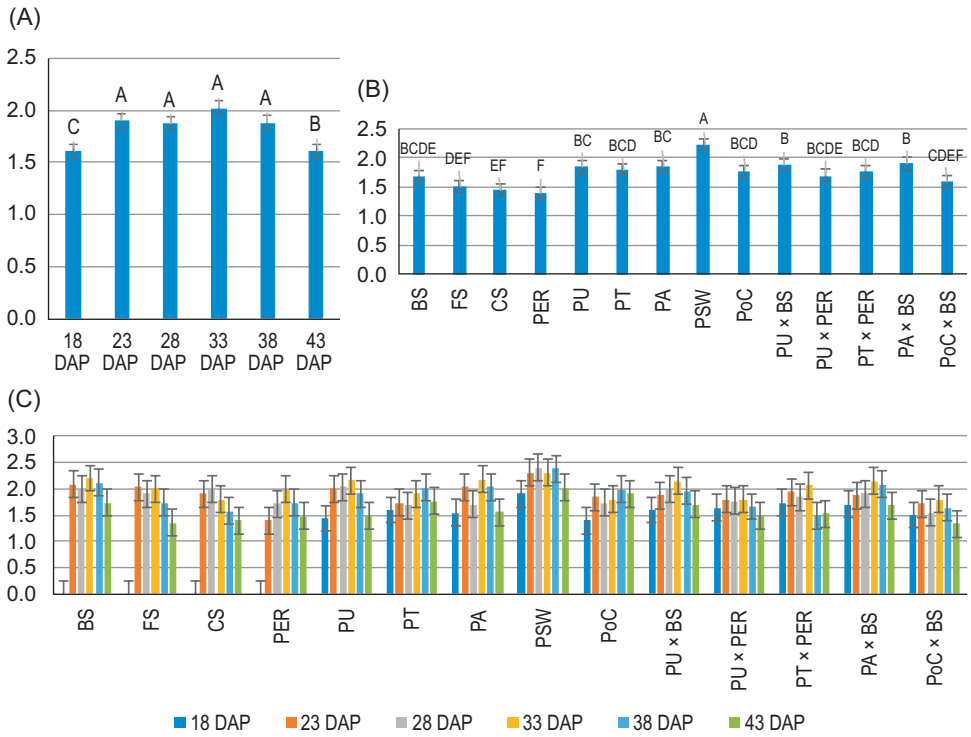

Fig. 2 Effect of ovule age on *in vitro* ovule growth (mm²) during incubation and maturation in various grape genotypes and their cross combinations.
(A) Ovule age; (B) Genotype; (C) Ovule age and genotype interaction. DAP, Days after pollination.
Treatment details are given under Materials and Methods.

Regardless of the genotype, ovule age had a significant impact on *in ovulo* embryo germination, the highest *in vitro* germination (25.47%) occurred at sapling stage 33 DAP, followed by 19.20% at 38 DAP, 16.41% at 28 DAP, 12.20% at 43 DAP, and 10.52% at 23 DAP. Conversely, the lowest germination rate (1.75%) was observed at 18 DAP ovule age. Genotype variations were also evident in germination rates. Genotype PA exhibited the highest mean *in vitro* germination (28.44%), followed closely by PER (27.31%), while CS had the lowest (6.46%). Among the cross combinations, PA × BS displayed the highest mean germination (18.57%), while

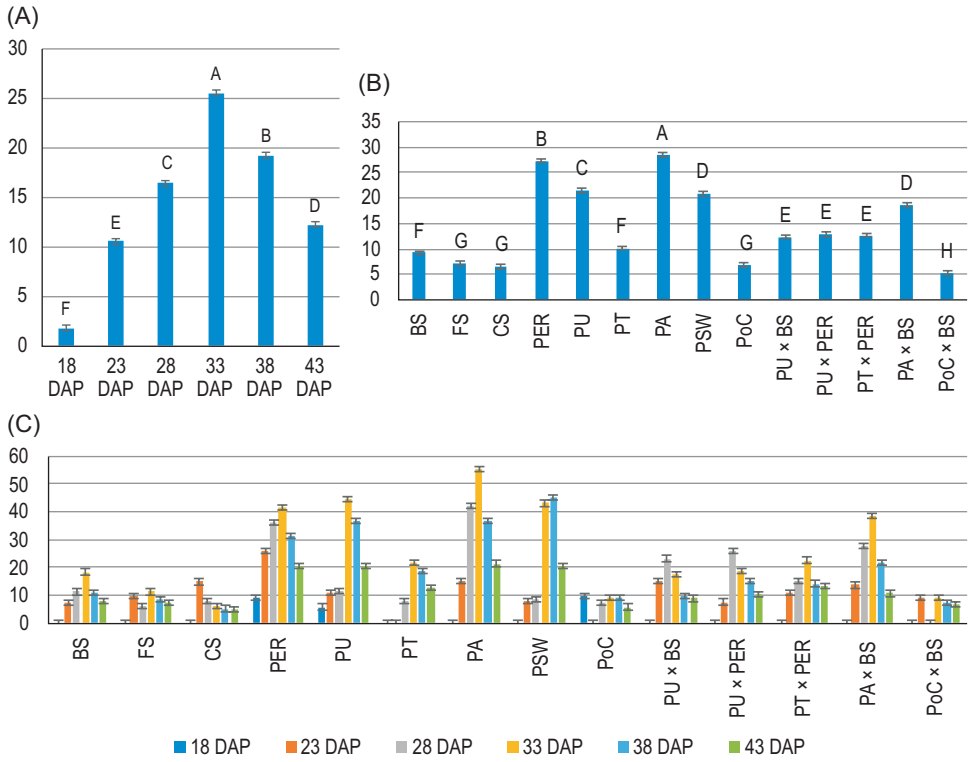

Fig. 3 Effect of ovule age on *in vitro in ovulo* embryo germination percent in various grape genotypes and their cross combinations.
(A) Ovule age; (B) Genotype; (C) Ovule age and genotype interaction. DAP, Days after pollination.
Treatment details are given under Materials and Methods.

PoC × BS registered the lowest (5.27%). Moreover, the optimal sampling time varied among the genotypes (Fig. 3). Notably, PA at 33 DAP ovule age showed the highest germination rate (55.19%), whereas PU genotype at 18 DAP ovule age had the lowest (5.71%). At 18 DAP, no embryo germination was observed (Fig. 3), except the lowest embryo survival rates were found for PER (9.01%), PoC (9.81%), and PU (5.71%). In our experiment, most genotypes displayed maximum germination at 33 DAP ovule age, except for cross combinations PU × PER (26.01%) and PU × BS (23.15%) at 28 DAP, CS (14.08%) at 23 DAP and PSW (45.04%) at 38 DAP ovule age. These findings align with Guo *et al.* (2011) research, which reported lower embryo germination

Table 2 Effect of ovule age on days to *in vitro* embryo germination in various grape genotypes and their cross combinations

Genotype/Cross combination	Ovule age (days after pollination)						
	18	23	28	33	38	43	Mean
BS (OP)	*	119.67	112.67	106.33	109.67	112.00	93.39^{F}
FS (OP)	*	132.00	128.67	123.33	122.67	126.00	105.44^{C}
CS (OP)	*	105.67	120.33	121.00	124.00	126.33	99.56^{D}
Per (OP)	132.33	124.00	101.00	88.33	94.33	97.67	106.28^{C}
PU (OP)	139.00	118.33	111.00	93.33	97.67	98.00	109.56^{B}
PT (OP)	*	0.00	126.33	111.33	116.67	118.33	78.78^{I}
PA (OP)	*	102.00	95.67	85.33	91.67	94.67	78.22^{I}
PSW (OP)	*	126.00	117.67	95.00	94.00	94.67	87.89^{G}
PoC	134.67	*	148.33	157.33	155.00	152.33	124.61^{A}
PU × BS	*	97.67	95.67	90.33	96.33	98.00	79.67^{HI}
PU × PER	*	94.33	89.67	85.33	86.67	90.33	74.39^{J}
PT × PER	*	93.33	88.00	83.00	85.33	83.00	72.11^{J}
PA × BS	*	107.00	101.33	91.33	94.33	92.67	81.11^{H}
PoC × BS	*	159.33	*	142.00	139.00	136.33	96.11^{H}
Mean	135.33^{A}	114.94^{B}	110.49^{C}	105.24^{F}	107.67^{D}	108.60^{E}	
Factor	DAP	Genotype (G)	G × DAP				
LSD ($P \leq 0.05$)	1.494	2.282	5.589				

*No germination. The values with different alphabetical letters differ significantly at 5%. The capital letters show differences among the mean value for both factors.

Treatment details are given under Materials and Methods.

rates at early stages and higher germination rates in the late germination stage, associated with torpedo-shaped embryos (Li *et al.* 2015).

Effect of ovule age on days to embryo germination: With increasing embryo age, survival and germination are often higher (Soni *et al.* 2019) since larger berries have bigger and more mature ovules that contain more viable embryos and have early germination capability (Liu *et al.* 2015). We examined the influence of ovule age on days to *in vitro* embryo germination (Table 2). It was found that when ovules cultured at a later stage of sampling exhibited early germination (Table 2).

Significantly the minimum (105.24) and maximum (135.33) number of days required for embryo germination irrespective of genotypes was recorded at 33 DAP and 18 DAP ovule age, respectively. A highly significant effect on days to germination irrespective of ovule age was also evident from the data. It was observed that the cross combinations PT × PER required the least number of days (72.11) and PoC × BS had a maximum germination time of 124.61 days. The interaction between the ovule age and genotype was also found significant. The minimum (83.00) number of days required for embryo germination was observed for PT × PER at 33 DAP and 43 DAP followed by 85.33 days in PA and PU × PER at 33 DAP ovule age and the maximum (159.33 days) for POC × BS at 23 DAP, although it was statistically at par to 157.33 days in PoC at 33 DAP ovule age. These results confirm the findings of Midani (2002), Liu *et al.* (2008), and Giancaspro *et al.* (2022) who reported the significant effect ovule culture on ovule survival and development.

To optimize the success of embryo rescue, it is crucial to effectively control embryo abortion and ensure precise timing for ovule culturing. With the sub-tropical region's short grape-growing season, the development of early seedless grape genotypes is of paramount importance, and embryo rescue offers a powerful tool to accelerate this process. Our findings highlight the significance of precise timing *in ovulo* embryo rescue with high recovery of hybrid progenies, the optimal ovule age for maximum germination is at 33 DAP, with exceptions being Centennial Seedless, the cross combination BS × PU and Pusa Swarnika where the suitable ovule ages were found to be 23 DAP, 28 DAP and 38 DAP, respectively. By harnessing this knowledge, we can enhance grapevine cultivation and grape improvement breeding programmes, ultimately contributing to the success of the viticulture industry in the sub-tropical region, which has a short crop cycle.

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