Stratification and SSR markers integration for promoting low chill peach (Prunus persica) hybridization in foot hills of Himalayas

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ABSTRACT

The present study was carried out at horticulture research centre, Patharchatta of G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand and Uttarakhand Council for Biotechnology, Haldi, Uttarakhand, during 2021 and 2022 with an objective to facilitate hybridization in low chill peaches [Prunus persica (L.) Stokes]. Selected cultivars of low chill peaches were crossbred using Saharanpur Prabhat, Sharbati Late and Sharbati Surkha as female parents, and Florida Red, Pratap and Early Grand as male. Among the hybrids, H2 (Saharanpur Prabhat × Pratap) showed the maximum fruit set (76.74%), while H5 (Sharbati Surkha × Pratap) had the minimum (55.93%). The highest fruit retention (71.15%) was noticed in H4 (Sharbati Late × Florida Red), and it was lowest in H2 (39.85%). H4 had the longest fruit development period (98.75 days), whereas H2 had the shortest (75.75 days). The seeds of hybrid H4 took the maximum days for stratification (58.75 days), while it was lowest in H2 (48.25 days). The germination percentage was estimated highest in H4 (83.88%) and lowest in H2 (65.99%). Out of 25 SSR markers tested for hybridity confirmation, only 3 (CPPCT-022, UDP96-005 and UDP98-407) were discovered to be capable of testing the hybridity of F1 seedlings. Results from this study will be helpful in improving the recovery of low chill peach hybrids and ensuring the hybridity of seedlings at a very early stage.

Keywords: Hybridity confirmation, Prunus persica, Summer stratification, SSR marker

Peaches [Prunus persica (L.) Stokes] are one of the most important fruits of the Rosaceae family and rank third after apples and pears. Though most opine that the peach originates in China (Wang 1985, Faust and Timon 1995), a few suggest that it originated in Persia. Hilly areas of Tibet and southwest China are the primary centers of peach diversity, while Iran is the secondary center (Kumar et al. 2013). The fruit of subtropical peaches are harvested nearly 1–2 months earlier than those grown in hills, resulting in good economic returns. In comparison to temperate regions, the fruit development period in the subtropics is quite short due to which the fruit ripening takes place before the embryo could complete its morphological and physiological development. It leads to embryo abortion and seeds are not adequately developed due to the little food reserves left in the embryo resulting in poor seed germination of hybrid, which is a major roadblock in the development of novel low chill peach hybrids.

In order to breed early ripening peach cultivars, embryo rescue is a valuable tool. It is required for the germination of embryos in cultivars that ripen in less than 110–120 days (Monet and Bassi 2008). However, in the subtropics, the hardening period of these embryo rescued plants coincides with the hot weather resulting in high mortality. Therefore, to accelerate the breeding cycle and maximize the survival percentage of hybrid seedlings, summer stratification under controlled conditions can also be done (Singh et al. 2017).

Molecular markers such as microsatellites or simple sequence repeats (SSRs) are abundant and widespread in plant’s genome, and due to their codominant inheritance, polymorphism, high discriminating power and relatively low cost, they have become an essential part of fruit breeding in the 21st century. They have been developed and used for diversity analysis (Trifonova et al. 2021) and also for parentage confirmation (Pandey et al. 2019). As a result of these considerations, the current experiment, consisting of summer stratification and SSR markers identification was conducted to maximize the success of low chill peach hybridization in the subtropics.

MATERIALS AND METHODS

The field study was carried out at the horticulture...
research centre, Patharchatta, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand during the spring season of 2021. The SSR genotyping for the hybridity test was carried out at Uttarakhand Council for Biotechnology, Haldwani, Pantnagar, Uttarakhand during 2022.

**Experimental material:** Different cultivars of selected low chill peach were crossed to develop the hybrids, viz. H₁ (Saharanpur Prabhat × Florida Red), H₂ (Saharanpur Prabhat × Pratap), H₃ (Saharanpur Prabhat × Early Grand), H₄ (Sharbati Late × Florida Red), H₅ (Sharbati Sukhha × Pratap) and H₆ (Sharbati Sukhha × Early Grand). Both the hybrids and the parents were observed with respect to data on fruit set, retention and fruit drop percentage. Fruits were collected separately after reaching full maturity. Then the seeds were extracted from the fruit, and summer stratification in the stratification media (2:1 ratio of cocopeat and perlite) under control conditions at 4±1°C. The optimum moisture content was maintained in the media and checked regularly. To prevent fungal infestation, the stratification media was moistened with 1% carbendazim. Seeds were correctly labelled and stored until the radicle emergence. When the seeds held for stratification, radicle emergence started, and thereafter the data on days to stratification were recorded. The seeds having emerged radicles were placed in rooting trays and kept in a growth chamber for germination, and the data on days to seed germination and germination percentage were noted. For further growth of the seedlings, rooting trays were transferred to high tech polyhouse having an optimum day (23–25°C) and night (20°C) temperatures.

**DNA extraction:** The newly emerged leaves from the parents and hybrid seedlings were collected. The CTAB method (Doyle and Doyle 1987) with minor modifications was used to extract the genomic DNA and the nano-drop spectrophotometer was used to determine the purity and quantity of DNA.

**Primers used and PCR amplification:** A set of 25 SSR primers (Supplementary Table 1) were selected based on their reproducibility and high discrimination power as observed in previous studies (Cipriani et al. 1999, Sosinski et al. 2000, Aranzana et al. 2002, Dirlewanger et al. 2002, Yamamoto et al. 2002). Fifty nanograms of template DNA, 0.2 mM total dNTPs, 0.5 μM primer, 1.0 μl of 10× PCR buffer, 1.5 mM magnesium chloride, and 1 unit of Taq polymerase were used to amplify DNA in 10 μl reactions. The DNA amplification included the following cycle: initial denaturation for 4 min at 94°C (1 cycle), then 35 cycles each of denaturation (94°C for 1 min), annealing (52–60°C for 1 min) and elongation (72°C for 1 min) then final extension at 72°C for 7 min (1 cycle). The amplified PCR product was mixed with 6x loading dye and then electrophoresed in 2.5% agarose gel containing ethidium bromide (1.0 μg/ml) in 0.5x TBE buffer. The reaction products were then visualized under a UV transilluminator and photographs were taken on a UV light gel documentation system.

**Hybridity confirmation and statistical analysis:** The SSR markers are expected to amplify alleles of both the parents and therefore visualized in the form of two amplicons in the hybrids whereas the parental lines will show their respective alleles in the form of a single amplicon. Based on the amplification pattern with a marker each hybrid was identified from its parental lines. The numeric data were subjected to Analyses of Variance (ANOVA). Significant differences among groups were determined using Duncan’s multiple range tests at P<0.05. All computation and statistical analyses were done using IBM SPSS Statistics 19 statistical software (IBM, NY, USA).

### RESULTS AND DISCUSSION

The data with regard to fruit set, drop and retention percentage in the parent cultivar (O.P.) are depicted in Table 1. The cultivar Sharbati Sukhha exhibited the lowest fruit set (46.50%) and the highest was noted for Sharbati Late (78.31%). The lowest fruit drop (38.66%) was observed in Pratap and the highest in Saharanpur Prabhat (60.43%). Inversely, the highest fruit retention was examined in Pratap (61.44%), and lowest in Saharanpur Prabhat (39.54%).

Results (Table 2) showed the significant difference in fruit set among all the 6 cross combinations. The highest fruit set (76.74%) was recorded in the hybrid H₂ (Saharanpur Prabhat × Pratap), which was statistically at par with H₅ (Sharbati Sukhha × Early Grand; 74.61%), while it was estimated lowest in H₁ (Sharbati Sukhha × Pratap; 55.93%), which differ non-significantly with H₅ (Sharbati Late × Florida Red; 61.67%). Fruit retention ranged from 39.85 to 71.15% being the highest in the hybrid H₄ (Sharbati Late × Florida Red; 71.15%) which differed non-significantly to H₃ (Saharanpur Prabhat × Early Grand; 67.50%) and the least fruit retention percentage was registered in H₄ (Saharanpur Prabhat × Pratap; 39.85%). Fruit drop and final fruit retention were inversely proportional to each other. The fruit drop percentage also differed significantly among all cross combinations and recorded a minimum (29.05%) in hybrid H₁ and highest (60.85%) in H₃ (Table 2).

Comparing to parents, relatively less fruit set was noted among the hybrid however, they showed more fruit retention in general. It can be clearly observed (Table 2)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Fruit set (%)</th>
<th>Fruit retention (%)</th>
<th>Fruit drop (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saharanpur Prabhat</td>
<td>78.31a</td>
<td>39.54a</td>
<td>60.43a</td>
</tr>
<tr>
<td>Pratap</td>
<td>61.50b</td>
<td>61.44b</td>
<td>38.66c</td>
</tr>
<tr>
<td>Early Pratap</td>
<td>54.27bc</td>
<td>53.43b</td>
<td>45.90bc</td>
</tr>
<tr>
<td>Florida Red</td>
<td>74.30a</td>
<td>42.76d</td>
<td>58.77b</td>
</tr>
<tr>
<td>Sharbati Late</td>
<td>77.35a</td>
<td>52.74bc</td>
<td>48.10b</td>
</tr>
<tr>
<td>Sharbati Sukhha</td>
<td>46.50c</td>
<td>45.72cd</td>
<td>54.26bc</td>
</tr>
<tr>
<td>SEm±</td>
<td>2.77</td>
<td>2.33</td>
<td>2.44</td>
</tr>
<tr>
<td>CD (P = 0.05)</td>
<td>8.36</td>
<td>7.02</td>
<td>7.36</td>
</tr>
</tbody>
</table>

*Means with the same letter within a column shows non-significant differences (at P≥0.05) as per Duncan’s multiple-range test.
that the highest fruit retention and lowest fruit drop were noted when Sharbati Late was used as a female parent. Several factors might have contributed to the varying degree of fruit set, fruit retention and fruit drop among the different cross combinations, such as weather conditions during artificial pollination as well as quantity and quality of pollen used. Furthermore, environmental stress may also cause the degeneration and abortion of the ovules, resulting in low fruit set and retention percentages (Nava et al. 2009). More or less similar variations with regard to fruit set, fruit retention and fruit drop were recorded by several researchers (Sharma and Verma 2014, Alves et al. 2018 and Devi et al. 2018). Pandey et al. (2019) made multiple crosses using different cultivars of early ripening low chill peaches and recorded the highest fruit set (59.28%) for the Flordaglo × Florida Crest cross and the lowest (38.56%) for Florida Crest × Florida Grand. The highest (74.50%) and lowest (41.27%) fruit retention were witnessed in Flordaglo and Devi × Florida Crest, respectively. Goswami (2020) revealed Earli Grande and Florida Prince to be the best combiner for hybridization breeding as they were examined to have maximum fruit set when used as a female parent (66.50% and 62.20%, respectively).

Data on the fruit development period, the number of days taken for stratification and germination and the germination percentage of the selected peach crosses are presented in Table 3. The number of days taken for germination, seeds obtained from Sharbati Late and Sharbati Surkha crosses took lesser time for germination (65.99%) in H4, noted as highest (83.88%) for the hybrid H4 and lowest (65.99%) in H2.

The fruit development period in any fruit crop is mainly dependent on the cultivar or genotype. This is an important parameter taken into consideration for distant marketing. The variation observed for the fruit development period corroborates the findings of Chaurasiya and Mishra (2017). Stratification of seeds is needed to break the dormancy of the embryo. A strong effect of embryo genotype on the chilling requirement of the seeds and the difference in stratification period might be due to the need for the different chilling duration in different genotypes (Bruckner et al. 2012). The findings concerning the number of days taken for stratification are in accordance with those of Eroglu et al. (2016). They stratified the seeds of different peach crosses without endocarp at 4–5°C for 40 to 90 days and reported differential responses of the crosses.

With respect to the number of days taken for germination, seeds obtained from Sharbati Late and Sharbati Surkha crosses took lesser time for germination which might be due to the longer fruit development period, where the matured seeds accumulate more dry matter in the seeds. Srivastava et al. (2021) collected fully mature seeds of low chill peach cultivars during June and studied them by sowing either directly in situ (during 1st week of December) or after the stratification treatment during February. Regardless of the sowing method, they estimated maximum seed germination in the peach variety Sharbati (35%). The author also concluded that harvesting of Sharbati and Pant Peach-1 preferably to be done during June and pre-sown stratification is necessary for better germination and growth of seedlings under the subtropical climate of Lucknow.
Prunus persica may be useful for ensuring the hybridity of seedlings at a general winter stratification. Furthermore, SSR genotyping under subtropical conditions which can accelerate the advantage for the peach hybrid breeding programme along with the application of growth regulators could be hollow stones formation, therefore stratifying low chill peaches in the later stage following harvesting causes (70–75 days). The shrinkage of a kernel in early maturing to seed germination than cultivars maturing very early periods (>90 days) may prove to be suitable parents for that low chill peach cultivars with longer fruit development periods had better seed germination.

In the present experiment, out of 25 SSR markers used for the hybridity testing, only 3 (CPPCT-022, UDP96-005 and UDP98-407) of them have been found to confirm the hybridity of the F1 seedlings as they showed the two amplicons in the hybrid seedlings, instead of alternate amplicon in the parental cultivars (Fig 1). The results confirm the authenticity of the peach crosse made and their further use for future breeding programmes. It is very difficult to identify hybrid seedlings produced from a cross between diverse parents when one of the parents has a convenient dominant feature (Pandey et al. 2019). The identification of these characters has been accomplished by various researchers using different methods. The most commonly used methods of detecting hybrids include morphological, chromatographic and isoenzymatic procedures, but none of these provides a perfect guarantee of recognizing hybridity (Tusa et al. 2002). Hence, molecular markers are being used, which often result in the exclusion of true hybrid seedlings from segregating populations. The use of molecular markers is a very useful aid in helping the breeders to give immediate results. Due to their co-dominant inheritance and the large number of alleles per locus, SSRs provide a more reliable method to determine parentage. Likewise, several other researchers have also used SSR markers to confirm the parentage of hybrids in different stone fruits (Liu et al. 2007, Pandey et al. 2019).

It can be concluded from the findings of the present study that low chill peach cultivars with longer fruit development periods (>90 days) may prove to be suitable parents for hybridization in subtropics owing to their better response to seed germination than cultivars maturing very early (70–75 days). The shrinkage of a kernel in early maturing peaches in the later stage following harvesting causes hollow stones formation, therefore stratifying low chill peach hybrid seeds in summer under controlled conditions along with the application of growth regulators could be advantageous for the peach hybrid breeding programme under subtropical conditions which can accelerate the growth of hybrid seedlings by a 1/2–1 year compared to general winter stratification. Furthermore, SSR genotyping may be useful for ensuring the hybridity of seedlings at a very early stage of seedling growth.

REFERENCES
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![Fig 1 Hybridity confirmation via SSR marker.](image)

M, 100 bp (DNA ladder); P1, Sharbati Late; P2, Florida Red; H1, Sharbati Late × Florida Red.

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