Genetic Fidelity Study of Micropropagated Plants of Kiwifruit (*Actinidia deliciosa* cv. Hayward) using SSR, ISSR and RAPD Markers

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Abstract

The shoot-tip and mature seed of Kiwifruit (*Actinidia deliciosa* cv Hayward) obtained from field grown mother plant were inoculated in various concentrations of benzyl amino purine (BAP), α-naphthaleneacetic acid (NAA), in the full strength of Murashige and Skoog (FMS) media for micropropagation. FMS supplemented with 1 mg/L BAP responded best for seed germination whereas, FMS supplemented by 1.5mg/LBAP and 0.5 mg/L NAA responded better for shoot-tip regeneration. Further, FMS supplemented by 1.5 mg/L BAP and 0.2 mg/L NAA media combination showed the best response for *in vitro* shoot proliferation from both shoot tip and callus culture proliferating an average of 6.7 and 13 shoots, with 2.7 and 3.3 cm of shoot length, respectively. The *in vitro* regenerated shoots showed the best *in vitro* rooting in FMS supplemented with 1 mg/L of IAA. During acclimatization, 85% of plants were successfully hardened in the acclimatization medium with 3:1:1:1/2 ratio of cocopeat: sand: soil and pearlite. Random amplified polymorphic DNA marker (RAPD), inter simple sequence repeat (ISSR) primers and simple Sequence repeat (SSR) markers evaluated the genetic stability of *in vitro* regenerated plants. The result obtained suggest that, the regeneration protocol can be used for *in vitro* propagation of *Actinidia deliciosa* cv Hayward for the commercial production of true-to-type plants and will help genetic transformation studies in future. The method developed in this study can substantially assist propagation *in vitro* and benefit economically valued fruit crop *Actinidia deliciosa* cv Hayward breeding programs.

Keywords: kiwifruit breeding; tissue culture; direct and indirect organogenesis; genetic homogeneity; molecular marker

Introduction

Kiwifruit (*Actinidia sp.*) belongs to the family Actinidiaceae and is a dioecious perennial vine and a cash crop with big potential for export in the international market. Being a member of the genus Actinidia and family Actinidiaceae, kiwifruit is botanically known as *Actinidia deliciosa* [A. Chev.] C.F. Liang and A.R. Ferguson variety deliciosa (Liang and Ferguson 1986). It can be grown in the warm temperate region of the world. It is originated in East Asia, with the highest genetic diversity in eastern China, the crop was domesticated and commercialized in New Zealand as Kiwifruit in the last century (Ferguson and Huang 2007). *A. chinensis* variety *chinensis* (golden–fleshed) *Actinidia deliciosa* var. *deliciosa* (green-flashed) and *Actinidia argute* (hardy kiwi) are commercially cultivated species out of more than 54 species of the genus *Actinidia* (Ferguson and Huang 2007; Wang and Gleave 2016). Due to the large fruit size, good flavor, novel flesh cover, variations in the harvest period, improved yield, growth habit, hermaphroditism, tolerance to adverse conditions and resistance to diseases, two species namely *A. deliciosa* and *A. chinensis* are widely used in kiwifruit breeding programs (Wang and Gleave 2016). This fruit has both nutritional and medicinal values because the fruit contains a high amount of vitamin C and minerals especially potassium, magnesium, copper, zinc, calcium, iron and phosphorous, vitamins such as folate, vitamin E, Vitamin K and antioxidants (Ferguson and Ferguson 2003; Gosal and Wani 2018; Yue et al. 2020). The fruit is consumed fresh and in processed form as jam, juice and syrups. Kiwi vines do best in deep, well-drained soils with the optimum soil pH of 5.5 to 6.0 and in elevations ranging from 1,000 to 2,500 m above sea

level (MASL). South face area with adequate irrigation facilities is suitable for its cultivation. The male plant grows more vigorously than its female counterpart. Male and female vines must be present in a block. Usually, one male plant is sufficient to pollinate 8 to 10 females (Atrya et al. 2020; Thapa and Karki 2020). With around 50.6% (2230.07 million tons) of the total kiwifruit production across the world, China grabs the 1st position succeeded by Italy, New Zealand, Iran and Chile (FAO, 2020).

In Nepal, two species *Actinidia strigose* and *Actinidia callosa* are found in wild state and commonly they are called *Thekifal* in local language. However, the cultivated species including *Actinidia deliciosa* are newly introduced and rapidly emerging as a commercial fruit crops in the mid-hills of the country (Adhakary 2014). It is believed that this fruit was introduced to Nepal in 1979/80 AD by Swiss in Chirikot and Jiri, Dolakha district, Central Nepal. Later, in 1986/87 AD, it was officially introduced to the Horticulture Development Project, Kirtipur. Later, in 1998/99 AD, some kiwifruit varieties were brought from the Himanchal Province, India and planted by ICMOD, Godavari, Nepal (Thapa and Karki 2020). Currently, at government level, several programs related to the training and production of Kiwifruit saplings are being conducted in three stations: Warm Temperate Horticulture Centre, Kritipur, Temperate Fruits Rootstock Development Centre, Dolakha and Temperate Horticulture Nursery Centre Daman, Makanpur, Nepal (Thapa and Karki 2020). Apart from this Prime Minister Agriculture Modernization Project has identified three districts (Illam, Dolakha, Soukhumbu) as kiwi zone in order to promote kiwi farming at commercial scale (Sharma, Thapa and Khatiwada 2020)

The total area, productive area, production and yield of Kiwifruit in Nepal are 2450 ha, 859ha, 5880.37 Mt, 6.8 Mt/ha, respectively (MOAD, 2020/21). However, current fruit production is not sufficient to meet fresh fruit demand which could be considered to the poor biotechnological research and development in the agriculture sector of Nepal and shortage of quality planting material. In the year, 2020/21, about 173 metric ton (Mt) of fresh kiwifruit has imported to country (Ministry of Agriculture and Livestock Development 2022). Therefore, the production of high-quality fruits that can fulfill the domestic fruit demand and compete with the international fruit market is the country's current need. In addition to fresh fruit, it has tremendous potential for processed value-added products including jam, juice, candy, wine jelly, etc. (Vaidya, Vaidya and Sharma 2006).

Kiwifruit can be propagated in several ways such as by seed, grafting, cutting (softwood and hardwood), or budding (Çelik, Zenginbal and Özcan 2018). The seed-grown plant has some drawbacks, including vigorous climbing vines, a long (4-5 years) juvenile period, the difficulty of sex determination of seedlings and genetic variation. Therefore, grafting the desired variety onto a seedling rootstock is a widely used commercial practice. However, seed germination in natural conditions has many problems such as seed dormancy and seasonal and climatic variations (Çelik et al. 2018; Windauer et al. 2016). Moreover, being a dioecious genus with multiple ploidy series within species kiwifruit possess challenges in breeding and conservation efforts (Debenham and Pathirana 2021; Ferguson and Huang 2007). Currently adopted conventional plant production techniques are tedious and time-consuming therefore, plant tissue culture is the best alternative (Nasib et al., 2008). Plant tissue culture for kiwifruit production was first reported using stem and root segment of *Actineda chinensis* by Harda (1975) and after that many studies have applied various method of micropropagation using varied range of explants and genotypes, such as stem segment for indirect embrogenesis in *A. chinensis* (Huang 1981), nodel segment and shoot tip in *A. deliciosa* (Deb and Gangmei), shoot tip culture in *A. deliciosa* [A. Chev.] var. *deliciosa* (Thakur et al. 2022), seed in *A. deliciosa* (Akbaş et al. 2007), stem segments with buds in *A. deliciosa* cultivar Guichang (Zhong et al. 2021).

Acclimatization success and getting true-to-type of plant are very challanging. In the process of tissue culture due to various biotic and abiotic factor, there is always the chances of somaclonal variation (Goyal et al. 2015). Thus, the genetic stability of *in vitro* propagated plants should be assessed as early as possible so that the deleterious effects that might be expressed later in growth do not result in having a severe economic impact (Marum et al. 2009). The genetic homogeneity amongst the micropropagated plants with mother plant in different crops has been assessed by PCR based molecular markers such as random amplified polymorphic DNA marker (RAPD), inter simple sequence repeat (ISSR) marker, simple sequcence repeat (SSR) marker and amplified fragment length polymorphism (AFLP) (Rai et al. 2012; Thakur et al. 2016; Oliya et al. 2021a; Sultana et al. 2022). Among all molecular markers, microsatellite or SSR markers have gained considerable importance in genetic fidelity assessment due to many desirable characteristics like high reproducibility, co-dominant inheritance nature, enormous extent of allelic diversity, high abundance in organisms and strong discriminatory power (Nookaraju and Agrawal 2012). Since,

microsatellite marker is species-specific, costly and time consuming ISSR and RAPD primers can be used as a suitable alternative to microsatellite markers in those plants where microsatellite markers have not yet been reported.

Therefore, the objectives of this study were (i) to develop the micro propagation protocol for both direct and indirect organogenesis of *A. deliciosa* cv. Hayward using shoot-tip as the initial starting material and (ii) to analyze the genetic fidelity of *in vitro* regenerates using RAPD, ISSR and SSR markers.

Materials and methods

Plant materials

The young shoot tip and mature seed of kiwifruit *Actinidia deliciosa* cv. Hayward available on the Warm Temperate Horticulture Centre (WTHC) Kirtipur, Kathmandu, Nepal was used as an explant.

Media preparation

The full strength of Murashige and Skoog basal medium (FMS) (Murashige and Skoog 1962) that was supplemented with 3% sucrose (w/v) and agar 0.8%(w/v) (Agar-Agar, Thermo Fisher Scientific, India) alone or in combination with cytokinin, 6-benzyl amino purine(BAP), auxin, α-naphthaleneacetic acid (NAA) and coconut water (CW). The pH of all media was adjusted to 5.8 with either 0.1 NaOH or HCl. Plant growth regulators (except IAA, which was added in the autoclaved media by filter sterilization) were added to the medium before adjustment of pH and sterilization. The culture bottles (of 300 ml capacity) with 50 ml of MS medium were sealed with a white transparent cap and autoclaved at 121 °C under 105 KPa pressure for 15 min.

Surface sterilization

The collected shoot-tip and seed were washed with 70% ethanol for 3 minutes and then, kept in the beakers with 1–2 drops of Tween-20 and detergent. The mouth of the beaker was covered with muslin cloths to avoid explant loss and washed under running tap water for 30 min before being transferred to a Laminar airflow cabinet where they were surface sterilized by submerging in 1.5 % sodium hypochlorite solution for 10 min followed by 70 % ethanol for 3 min and finally rinsed with sterile distilled water three times.

Shoot-tip and seed inoculation of field grown plant

The surface-sterilized shoot-tips and seeds were kept on filter paper in the Petri plate to soak water and then, shoot-tip were inoculated in ten different treatments FMS, FMS in combination with (0.5, 1, 1.5 and 2) mg/L BAP and 0.2 mg/L of NAA, four shoots were placed in one culture bottle and six such replicates were made for each treatment group. *In vitro* responses for the proliferation of shoot, root and callus were examined after 8 weeks of culture.

Callus regeneration and in vitro developed shoot-tip culture

For shoot multiplication, the shoot tip and embryogenic callus that was developed *in vitro* from the shoot-tip culture (taken from ex vivo grown plant) were excised and inoculated separately in full strength (FMS) and half strength (HMS) of MS medium alone (control) and in combination with different concentrations of plant growth regulators such as BAP alone or in a combination of NAA and kinetin (Kn) and coconut water (CW) (Table 1). A total of 38 media treatments were used (Table 1). For shoot tip culture, two shoots were inoculated in one culture bottle and three such replicates were made. For callus culture, four calluses were inoculated in one culture bottle and 3 such bottles were made for each media treatment. The explants were examined for shoot proliferation after 10 weeks of culture.

Leaf petiole and node culture

From the shoot tip culture and callus regeneration experiment, M11 was found to be the best medium for the proliferation of the maximum number of shoots than other media used. Therefore, M11 has been used for leaf petiole and node culture experiments. Four explants were inoculated in one culture bottle and three replicates were made making a total of 12 replicates per treatment (Fig. 4F)

***In vitro* Rooting**

In vitro developed healthy shoots devoid of callus were transferred to FMS and HMS media supplemented with 0.5,1,2,3 and 4 mg/L IAA and IBA separately (Table 2). FMS and HMS devoid of IAA and IBA were used as control (Fig 5, 7A-D). Two shoots were inoculated in one culture bottle and three such culture bottles were used making 6 shoots per medium treatment. Explants were examined for root production after 6 weeks of culture.

Hardening and acclimatization

In vitro rooted shoots were washed to remove agar. The root was dipped in the 1mg/L of the antifungal agent bevistin (carbendazim 50% WP) solution for 2 min. Thirteen different medium treatments (Table 3) were made. Each medium was autoclaved and filled in a plastic cup. The individual shoot was planted in one plastic cup. The plastic cup was covered with transparent plastic and kept at room temperature with natural light supplement to maintain high humidity. After two weeks, the transparent plastic cover was removed gradually to reduce humidity. The potted plants were irrigated every four alternative days with half strength of MS liquid medium containing 1mg/L IAA but devoid of sucrose. To avoid the fungal infection, 1mg/L of Carbendazim 50% WP was applied weekly as a foliar spray for one month. After one month of hardening, the plants were transplanted to the glasshouse during Winter (November-March) and in the shed net house for the rest of the months (April-October). A total of 20 *in vitro* rooted plants were planted in each treatment and the experiment was replicated four times making 80 plants per treatment group.

Genetic fidelity study

Sample information

A total of 13 samples including one mother plant 12 plants regenerated from shoot tip culture on 1.5 mg/L BAP and 0.2 mg/L NAA and rooted on 1mg/L IAA and acclimatized on 3:1:1:1/2 ratio of cocopeat: sand: soil and pearlite were used for genetic fidelity study. The leaf of the individual plants was harvested after six month of acclimatization.

DNA isolation

DNA was isolated using the protocol of Doyle (1991) with modification. In this method, approximately 0.2g of leaf sample was taken and ground to fine powder by using a mortar and pestle in liquid nitrogen. After that, 1mL of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M tris HCL, 0.2% B-mercaptoethanol) was added to make a fine paste and transferred to a clean microcentrifuge tube (vol. 1.5µl). Then, samples were incubated at 65 ºC for 45 min in a water bath with gentle mixing at 10 min intervals. After incubation samples were centrifuged at 12,000 rpm for 8 min at room temperature to spin down cell debris. Then the supernatant was transferred to a clean sterilized microcentrifuge tube (vol. 2 uL). An equal volume of Chloroform: isoamyl alcohol (24:1) was added and mixed gently in orbital shaker for 8 min. It was again centrifuged for 5 min at 13,000 rpm and the upper aqueous phase was transferred to a sterile microcentrifuge tube. Approximately, 50 ul (1/10th volume of supernatant) of sodium acetate (3M), was added to each sample followed by the addition of 500 µl of chilled ice-cold absolute ethanol. The tubes were slowly inverted several times to precipitate the DNA. DNA was spinned at 13,000 rpm for 2 minutes to form the pellet. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol (500 µl volume). Again it was spinned at 13,000 rpm for 1 minute to get rid of salt. Then ethanol was pipetted out and the pellet was left to dry for 30 min at 37ºC. Then it was suspended in TE buffer (100 µL) and stored at 4 ºC. The quality of extracted DNA was estimated by electrophoresis on agarose gel (1%) and visualized in UV light in a Bio-gel documentation system (gelLITEPROSAFE, Cleaver Scientific, UK). DNA samples were stored at -20 ºC for downstream uses.

PCR amplification, RAPD, ISSR and SSR analysis

For the PCR amplification, a total of 15 ISSR, 15 RAPD and 15 SSR primers were used. Among them, 9 ISSR, 8 RAPD and 7 SSR primers that provided very clear and reproducible bands were used for genetic comparison. PCR amplification was performed on a final volume of 15 µl containing 30 ng of genomic DNA, 2X PCR master mix with dye (included 1× PCR buffer, 160 µM of each of the dNTPs, 0.8 U of Taq DNA polymerase and 6X loading dye (MedChem express, USA) and 0.5 µM each primer. The thermal cycling condition was carried out in a (Techne, TC-312, Japan) programmed at 95°C for 5 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing (at 47 °C for ISSR, 37°C for RAPD and 55°C for SSR primer) for 1m, extension at 72 °C for 2 m and

then a final extension at 72 °C for 10 min. To test the utility of the primers, PCR products were detected on 2% agarose gels and visualized by staining with ethidium bromide (0.5ug/ml) in a 1X TBE buffer at 95 V. PCR products were visualized on U.V. light and photographed using a Gel Documentation System (gelLITE PROSAFE, claver scientific, UK). Only the clear and reproducible amplicon was considered for analysis.

Laboratory information

Whole the experiment was conducted on the biotechnology Laboratory of Warm Temperate Horticulture Centre, Kirtipur, Kathmandu, Nepal. This Laboratory was established during the period of Horticulture Development Project (in 1985-1997) with the support of Japanese government however, remain non-operating for longer time and re-established for plant tissue culture in 2019 AD and Molecular testing system in 2022 AD.

Statistical analysis

All the data were analyzed using one-way analysis of variance (ANOVA) and the mean values for each parameter were compared for a significant difference using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The results were expressed as means ± standard error for each experiment. All statistical analyses were performed using the SPSS computer software (version 23; IBM, Armonk, New York).

Results

Shoot-tip inoculation of field grown plant

Among several media used, FMS supplemented with 1mg/L BAP and 0.5mg/L NAA responded best for shoot-tip (taken from ex-vivo plant) regeneration (75.0 %) and direct shoot proliferation (66%). On this medium only 10 % shoots responded for callus induction. FMS medium combined with 1.0 mg/L BAP responded second-highest response where, 53% of the explants showed response. Among the responded explant, 53% regenerated direct shoots whereas, 12 % explants induced callus. Similarly, on FMS combined with 1.5 mg/L of BAP 43% of the total inoculated shoots showed growth response. Among them, 33% explant developed direct shoots and 20 % explants developed callus. Similarly, FMS combined with 0.5, or 1mg/L BAP and 0.2 mg/L NAA responded better for callus induction (20%) and lower response for shoot tip induction 13% and 12%, respectively). The minimal response for shoot-tip culture was observed on FMS medium devoid of growth hormones (control) (Fig. 1, Fig.4 A-C).

Seed culture of field grown plant

Among the media used, on FMS medium combined with 1mg/L of BAP, 53% of the seed were responded. Among the responded seeds, 54% induced direct shoots. Least seed germination was observed on control (FMS devoid of growth hormones).

Callus regeneration

Among 38 media combinations used (Table 1), only 22 media regenerated shoots from the callus. Among them, M11 regenerated a significantly higher number of shoots (6.7 number average shoots) with the longest shoot length (average shoot of 2.7cm), followed by M10 proliferating 3.5 number of shoots with 2.2cm of shoot length. Similarly, M30, M8 and M31 regenerate 3.5, 2.5 and 2.2 numbers of shoots with 2, 0.5 and 1.5cm of average shoot length accounting for 3rd, 4$^{th \text{ and}}$ 5th highest callus regeneration, respectively. Other media regenerated no or little shoot. Maximum callus enlargement was found on M37 followed by M15, M17, M36, M38, M6, M8, M14 and M31 and the minimum or negligible callus growth was obtained on M1, M2, M3, M4, M5 and M30 (Fig. 2A-C, Fig.4 D)

In vitro developed Shoot tip culture

Among 38 media combinations (Table 2), M11 proliferated significantly higher (13) a number of shoots with the longest (3.3 cm) shoot length followed by M30 (11 shoots with 2 cm of shoot length), M10 (9 shoots having 2.3 cm shoot length, M29 (8 shoots, 2cm shoot length), M31 (6 shoots, 2 cm shoot length) and M32 (5 shoots, 2 cm shoot length). This media also supported the petiole and node culture producing multiple healthy shoots (5-7) with an average of 2.5 -3.5 cm of shoot length (Fig.4 A, B).

In vitro rooting

For the *in vitro* rooting of *Actinidia deliciosa,* a total of 21 media treatments (Table 3) were used. Among them, rooting media R2 and R12 were the best but significantly not different for the induction of more root numbers (16.0

and 15.7 roots, respectively). However, the root length of M2 (8.25 cm) was significantly longer than roots developed on M12 (7.56). Medium R3 proliferated 10.50 numbers of roots followed by M13 and M4. Higher concentration of IAA and IBA induced callus and poor roots. Compared to IAA, the roots induced in IBA had more fibrous roots but favored callus induction (Fig.5A, B).

Hardening and acclimatization

Among 15 different acclimatization media used (Table 3), media AC9 and AC10 were found to be most effective for primary hardening, giving significantly highest survival of 85% and 80%, respectively followed by AC11, AC12, AC6, AC4 and AC13 giving 47, 40, 31,27 and 25% plant survival. The least survival was observed on AC1 (Sand (3): soil (1), where 10% of the acclimatized plant survived. None of the plants survived in AC14 and AC15 (sand (1): soil (1) without soil sterilization (Fig. 6, Fig. 8). Also, the root developed on IAA-containing medium showed more survival rate in the process of acclimatization than that developed on IBA treated medium. The well-adapted plants in the primary hardening zone showed more than 95 % survival in the glasshouse during the winter season and more than 80% survival in the shed net house during summer (Fig. 7, Fig. 8).

Genetic fidelity study

Among the ten RAPD primers used only six primers have provided clear and reproducible bands ranging from 250 bp to 1500 bp. The number of scorable bands, for each primer, varied from two to six with an average of 4.1 bands per primer. Similarly, among ten, nine ISSR primers provided a total of 30 clear and reproducible amplification sizes ranging from 300 (UBC -825) to 1500 (ISSR-886) base pairs. The allele size per primer ranged from two (ISSR-889) to six (ISSR-890). All the seven SSR primers that were used for the genetic fidelity study amplified reproducible and clear bands ranging from 180 bp (Ke115) to 350 bp (Ke112), resulting in the amplification of a single band (allele), corresponding homozygous individuals. All three DNA markers RAPD, ISSR and SSR provided monomorphic bands across the *in vitro* regenerates and with the mother plant (wild type) suggesting genetic stability of *in vitro* propagated plants (Table 4, Fig. 9)

Discussions

The quality sapling demand for *Actinidia spp*. is increasing worldwide (Sedaghathoor and Noie 2016; Strik and Davis 2021). Therefore, mass-scale propagation of kiwifruit is the most quickly and economically viable method as it can be produced irrespective of the season and planting material such as seed, genotype, explants, provided nutrient, light, pH and humidity plays an important role in the success of tissue culture (Famiani et al.1997; Holmes et al. 2021; Oliya et al, 2021). In the present study, MS medium supplemented with a low concentration of BAP (0.5, 1 and 1.5) mg/L was better for producing a large number of healthy green shoots compared to 2 mg/L BAP which showed decreased performance for induction of both shoot and highly regenerative callus from seed culture. Previously, Akbaş et al. (2007) used various strengths of MS medium (full, half, quarter and double) supplemented by multiple carbon sources (sucrose, maltose and dextrose at 3% concentration), BAP or Kinetin (0.5,1,2 and 4) mg/L BAP for *in vitro* mature seed culture of Kiwifruit and found full strength of MS medium supplemented by 3% sucrose and combined with lower BAP concentration (0.5 and 1) mg/L better for the proliferation of a significantly large number of healthy green shoots. The present study found MS medium combined with 0.5 or 1 mg/L BAP and 0.2 mg/L NAA, or FMS combined with 2 mg/L BAP better for callus regeneration. In support to our findings, MS medium supplemented by the higher concentration of NAA showed better performance for induction of callus from leaf disc obtained from field-grown *Actinidia chinensis* Planch. var. *hispida* 'Hayward' (Suezawa et al., 1988).

In the present study, FMS medium combined with 1.5 mg/L BAP and 0.2 mg/L NAA stimulated significantly higher responses for callus regeneration and shoot tip proliferation. This media also supported leaf pedicel and node culture. On this media, the growths of regenerated plants were very good with the multiplication of the morphologically similar highest number of shoots longer shoots in comparison to other media used. In support of the present findings, green healthy shoot proliferation of *A. chinensis* from axillary buds was favored on the full strength of MS medium supplemented with BA or zeatin compared to half strength of the same medium (Shen et al. 1990). Previously, shoot multiplication from axillary buds of the Alison variety of kiwifruit was achieved on a medium supplemented with 1 mg/L BAP together with 0.1 mg/L IAA (Kumar and Sharma 2002).

Among the two auxins applied for *in vitro* rooting, FMS supplemented with 1 mg/L IAA responded significantly better for both root induction and elongation. Furthermore, the root developed with IAA has a longer root length, no callus growth and a better survival rate in acclimatization than those developed on the same concentration of IBA. In support of the present finding, MS medium containing 1 mg/L IAA was better for *in vitro* rooting of kiwifruit (Kumar et al. 1998). Previously, Akbaş et al., (2007) reported 1mg/L NAA better for *in vitro* rooting of kiwifruit shoots. Rooting of kiwifruit shoots has usually been achieved by soaking or immersion in an IBA solution followed by culture on a rooting medium or by direct transplanting to a potting mixture (Kumar and Sharma 2002).

The success of tissue culture depends on acclimatization success as the sufficient number of micropropagated plantlets of several species fail to survive during lab-to-land transfer making use of this technique limited for many important plant species (Chandra et al. 2010; da Silva et al. 2017; Oliya et al. 2021b). Anatomical and physiological transition is also important for hardening while transferring plantlets from *in vitro* to *ex vitro* environments. The poor survival of *in vitro* raised plants under *ex vivo* conditions is due to soft green leaves and stems with high moisture, large spaces between their palisade cells and few stomata, poor cuticular and root development, a high degree of transpiration and susceptibility to pathogens (Bag et al. 2019; Oliya et al. 2021b). Therefore, hardening of *in vitro* raised plantlets may require special infrastructure, like a greenhouse, soil sterilization system, misting chamber, fogging, UV-treated water, etc. and is a time-consuming, labor-intensive procedure, adding to the production costs. At the time of hardening, micropropagated plants slowly adapt to the climatic conditions in the *ex vivo* environment, develop the ability to cope with the attack of various microorganisms and also develop functional photosynthetic machinery and other physiological systems (Deb and Imchen 2010; Khajuria et al., 2020). Under such conditions, plants convert rapidly from a heterotrophic or photo mixotrophic state to an autotrophic growth, develop a fully functional root system and better control their stomatal and circular transpiration required for survival after the lab-to-land transfer (Dhawan and Bhojwani 1987; Chandra et al. 2010). Therefore, hardening and acclimatization of kiwifruit require more attention as hairless thin roots produced *in vitro* die shortly after transplanting to the soil, hindering acclimatization success (Kumar and Sharma 2002).

Previously, a one-month-old shoot developed from the callus culture of *Actinidiasp.* were treated with fungicides, dipped in a concentrated solution of IBA and placed in glass jars on a peat-perlite mixture for the rooting phase, acclimatization was achieved gradually by opening the jar lid every day for one week, after that the plantlets were transferred to soil (Barbieri and Morini 1987). Similarly, in the report of Akbas et al. (2007), *in vitro* rooted shoots of kiwifruit were acclimatized in 1:1 mixture of sand and soil. However, in the cited literature there is a lack of sufficient supporting data and a suitable protocol. The present study has observed the acclimatization behavior of *in vitro* rooted plants of *A. deliciosa* on a large number of acclimatization media with strong supporting data and methods. Our study found the use of cocopeat or soil alone and without treatment of an antifungal agent was not good to support the *in vitro* rooted kiwifruit. However, the media (mixture of one part of soil, one part of sand, three parts of cocopeat and half part of pearlite) is found best for the primary hardening of kiwifruit. It is very important to maintain the light intensity, temperature and soil sterilization followed by periodical reduction of humidity and application of reduced concentration of MS nutrients in primary hardening to get maximum acclimatization. Furthermore, the plants having a strong stem and profuse root system showed acclimatization more successful in comparison to those having weak stems (reduced stem diameter, reduced leaf width) and poor root systems (less number of roots and induction of callus). This study found optimal primary hardening success in the control room where the temperature and the light intensity were maintained as that was in the growth room (light intensity =1800 lux and the temperature = 24 ± 2). The well-acclimatized plant showed elongated shoots, wide leaf breath and the formation of new leaves and enlargement of stem diameter which certain the formation of new roots in the substrate. However, primary hardening in normal room conditions was highly affected by temperature fluctuation leading high mortality rate of acclimatized plants. The well-adapted plants in the primary hardening showed more than 90% survival in the glass house during the cold season (November-March) and more than 80 % in the shednethouse during summer (April-October). It is also suggested for soil sterilization and fumigation of glasshouse and shednethouse to maintain a sterile environment and reduce the plant mortality rate from the fungal infection.

The genetic integrity of the plants derived through micropropagation becomes crucial if genetic transformation studies have to be carried out. It is well known that callogenesis is a source of additional variability in plant tissue cultures. The presence of somaclonal variation in populations derived from tissue culture is affecting the use of

tissue culture negatively and has remained a major problem. Conversely, it is a source of new desirable clones/variants with better agronomic traits (Bairu et al. 2011). In the present study, one wild (mother plant) and twelve plants that were developed from shoot tip culture on MS medium supplemented by 1.5 mg/L BAP and 0.2 mg/L IAA and successfully acclimatized on 3:1:1:1/2 ratio of cocopeat: soil: sand: and pearlite were used for genetic fidelity study using three DNA marker system RAPD, ISSR and SSR. All three marker systems evaluated the genetic stability of *in vitro* plants developed plants. Among three marker systems, all the applied SSR markers, 90% of ISSR markers and 70% of the RAPD primers generated reproducible and clear bands suggesting more applicability of SSR and ISSR than RAPD in genetic study.

Genetic fidelity studies of micro-propagated plants of several crops have been evaluated by DNA based molecular markers such as RAPD, ISSR, SSR either individually or in combined. Using ISSR marker Thakur et al. (2012), evaluated the genetic homogeneity of *in vitro* regenerants of *A. deliciosa* [A. Chev.] var. *deliciosa* developed from shoot tip culture. Rai et al. (2012) evaluated the genetic homogeneity of 1-year-old guava (*Psidium guajava* L.) plants developed from *in vitro* somatic embryogenesis using ISSR and RAPD primers. Qahtan et al. (2022) reported the genetic stability of callus-regenerated plants of *Ruta chalepensis* L using ISSR marker. Oliya et al. (2021) using RAPD primers reported 100% genetic homogeneity of *in vitro* developed red-listed endangered Orchid *Rhynchostylish retusa,* that were developed from immature seed culture. Similarly, using ISSR and SSR primers, Nookaraju and Agrawal (2012) reported 100% genetic homogeneity of six-month-old tissue culture-raised plants of grapevine cv. Crimson seedless, that were developed from single node culture. Saha et al. (2016) using RAPD and ISSR primer detected genetic stability of *in vitro* developed clones of *Morus alba* L. variety S-1 developed from node culture. ISSR markers showed the monomorphic banding pattern of the shoot tip and nodal segment grown micro propagated plants of periwinkle (*Catharanthus roseus* L.). The findings of the present study imply that the newly developed protocol is suitable for large-scale production of true-to-type plants. In contrast to our result, the somaclonal variation in the callus derived *in vitro* propagated plants have been reported by Ali et al. (2019) in *Orthosiphon stamineus* Benth. Using direct somatic embryogenesis, up to 55% somaclonal variation in the regenerated tea plant (*Camellia sinensis*) has been reported (Thomas et al. 2006). Bello-Bello et al. (2014) reported ISSR polymorphism in Habanero pepper (*Capsicum chinense* Jacq.) regeneration.

Conclusions

This study has established an efficient micropropagation protocol for both direct and indirect organogenesis using shoot-tip as the starting material of Kiwifruit (*Actinidia deliciosa* cv Hayward) and evaluated the genetic stability of *in vitro* regenerated plants using RAPD, ISSR and SSR markers. To the best of our knowledge, this is the first report for micropropagation of kiwifruit in Nepal. The result obtained suggests that our regeneration protocol can be used for *in vitro* propagation of *Actinidia deliciosa* cv Hayward for the commercial production of true-to-type plants and genetic transformation studies in the future. This protocol will help to supply large number of quality saplings (mother plants) for commercial cultivation of kiwifruit which is expected to replace the conventional plant production approach. Moreover, the use of healthy rootstock for plant production minimizes the cost of production and increase the productivity, which will help to meet the supply constrain and economic development of the country. Therefore, the high percentage of regeneration, good restoration of the plant in nature and genetic stability by combined marker system are reliable reasons to suggest that the method can substantially assist propagation *in vitro* and benefit economically valued fruit crop *Actinidia deliciosa* breeding programs. In addition, the established tissue culture protocol for the kiwifruit would be base for the establishment of micropropagation system for other fruits.

Compliance with Ethical Standards

Conflicts of interest

All authors declare that the authors and their immediate family members do not have any potential conflict of interest regarding this study.

Ethical approval

Not applicable

Informed Consent

The research does not involve human participants and/or animals

References

Adhakary, B. H. (2014). *Production Technology for Kiwi-Fruit*. Lalitpur: Nepal Agriculture Research Council (NARC).

Akbaş, F. A., Işikalan, C., Namli, S. Ü. R. E. Y. Y. A., & Başaran, D. A. V. U. T. (2007). Micropropagation of kiwifruit (*Actinidia deliciosa*). *International Journal of Agriculture and Biology*, *9(3),* 489-493.

Ali, H., Musa, I. F., Abu Bakar, N. A., Karsani, S. A., & Yaacob, J. S. (2019). *In vitro* regeneration and ISSR-based genetic fidelity analysis of Orthosiphon stamineus Benth. *Agronomy*, 9(12), 778.

Atreya, P. N., Shrestha, C. M., Suvedi, B. D., & Pandey, S. P. (2020). Emerging fruits of Nepal: pomegranate, kiwifruit, avocado, dragon fruit and grape; opportunities, challenges and ways forward. In *Proceeding of 11th National Horticulture Seminar* (pp. 6-7).

Bag N., Palni, L.M.S. Nandi, S.K. (2019) An efficient method for acclimatization: *in vitro* hardening of tissue culture-raised tea plants (*Camellia sinensis* (L.) O. Kuntze). *Curr Sci 117 (2):*288–293.

Bairu, M.W., Aremu, A.O., Van S. J. (2011). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation.63(2*):147-73.

Barbieri, C., Morini, S. (1987). Plant regeneration from *Actinidia* callus cultures. *J Hort Sci, 62(1)*: 107–109.

Bello-Bello, J.J, Iglesias-Andreu, L.G., Avilés-Vinas, S.A., Gómez-Uc, E., Canto-Flick, A., Santana-Buzzy, N. (2014). Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. *Hort Science, 49:* 481–485.

Çelik, H., Zenginbal, H., Özcan, M. (2018). Enhancing germination of kiwifruit seeds with temperature, medium and gibberellic acid. *Hort Science 33:* 39–45. https://doi.org/10.17221/3738-hortsci

Chandra, S., Bandopadhyay, R., Kumar, V., Chandra, R. (2010). Acclimatization of tissue cultured plantlets: from laboratory to land. *Biotechnol Lett 32,* 1199–1205.

da Silva, J.A.T., Hossain, M.M., Sharma, M., Dobránszki, J., Cardoso, J.C., Songjun, Z. (2017) Acclimatization of *in vitro* derived *Dendrobium*. *Hort Plant J 3 (3),* 110–124.

Deb, C. R., Gangmei, P.K. (2019). *In vitro* propagation of Kiwi from nodal segments and development, a new rooting technique of micro shoots. *Biotechnol 18*, 64–76 10.3923/biotech.2019.64.76

Deb, C.R., Imchen, T. (2010) An efficient *in vitro* hardening technique of tissue culture-raised plants. *Biotechnol 9,* 79–83.

Dhawan, V., & Bhojwani, S. S. (1987). Hardening in vitro and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of Leucaena leucocephala (Lam.) de Wit. *Plant Science, 53(1)*, 65-72.

Famiani, F., Ferradini, N., Standardi, A., Hoza, D., Stanica, F., (1997) *In vitro* regeneration of different *Actinida sp. Acta Horticult 444*: 133–138. https://doi.org/10.17660/Acta Hortic1997

FAO (2020). Food and Agriculture Organization of the United Nations. FAOSTAT statistical database, Rome. http://www.fao.org/faostat. Accessed 20 Nov 2022

Ferguson, A.R., Ferguson, L.R. (2003). Are kiwifruit really good for you? *Acta Horticult 610:* 131–138. https://doi.org/10.17660/ActaHortic.2003.610.16

Ferguson, A. R., & Huang, H. (2007). Genetic resources of kiwifruit: domestication and breeding. *Horticultural reviews, 33*, 1-121. https://doi.org/10.1002/9780470168011.ch1

Gosal, S.S., Wani, S.H. (2018) Biotechnologies of Crop Improvement. *Biotechnol Crop Improv 1* 1–497. https://doi.org/10.1007/978-3-319-78283-6

Goyal, A. K., Pradhan, S., Basistha, B.C., Sen, A. (2015) Micropropagation and assessment of genetic fidelity of *Dendrocalamus strictus* (Roxb.) nees using RAPD and ISSR markers. *3 Biotech 5(4)* 473–482. https://doi.org/10.1007/s13205-014-0244-7

Harada, H. (1975) *In vitro* organ culture of *Actinidia chinensis* PL as a technique for vegetative multiplication . *Journal of Horticulture Science 50* 81–83. https://doi.org/10.1080/00221589.1975.11514606

Holmes, J. E., Lung, S., Collyer, D., & Punja, Z. K. (2021). Variables Affecting Shoot Growth and Plantlet Recovery in Tissue Cultures of Drug-Type *Cannabis sativa* L. *Frontiers in plant science, 12,* 732344.

Huang, Z.G., Tan, C.Y. (1988) *Chinese gooseberry, kiwifruit* (*Actinidia* spp.). In Crops II (pp. 166-180). Springer, Berlin, Heidelberg.

Jain, S.M. (2001) Tissue culture-derived variation in crop improvement. *Euphytica 118,* 153–166

Khajuria, A. K., Bisht, N. S., & Bhagat, N. (2020). *In vitro* organogenesis and plant regeneration of *Thymus serpyllum* L.: an important aromatic medicinal plant. *In Vitro Cellular & Developmental Biology-Plant*, 56, 652-661.

Kumar, S., Chander, S., Gupta, H., Sharma, D.R., (1998) Micropropagation of *Actinida deliciosa* from axillary bud. *Phytomorphology 48*, 303-307

Kumar, S., Sharma, D.R. (2002.) *In vitro* propagation of kiwifruit*. Journal of Horticulture Science and Biotechnology 77*, 503–508. https://doi.org/10.1080/14620316.2002.11511530

Ladaniya, M. S., Marathe, R. A., Das, A. K., Rao, C. N., Huchche, A. D., Shirgure, P. S., & Murkute, A. A. (2020). High density planting studies in acid lime (*Citrus aurantifolia* Swingle). *Scientia Horticulturae, 261*, 108935.

Liang, C.F., Ferguson, A.R. (1986). The botanical nomenclature of the kiwifruit and related taxa. *N Z J Bot 24,*183–184

Chou-Fen, L., & Ferguson, A. R. (1986). The botanical nomenclature of the kiwifruit and related taxa. New *Zealand journal of botany, 24(1)*, 183-184.

Li, Q., Yu, P., Lai, J., & Gu, M. (2021). Micropropagation of the potential blueberry rootstock—Vaccinium arboreum through axillary shoot proliferation. *Scientia Horticulturae, 280,* 109908.

Marum, L., Rocheta, M., Maroco, J., Oliveira, M. M., & Miguel, C. (2009). Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*). *Plant Cell Reports, 28*, 673-682.

Mezzetti, B., Rosati, P., & Casalicchio, G. (1991). Actinidia deliciosa CF Liang in vitro: I. Growth and mineral uptake by explants. *Plant Cell, Tissue and Organ Culture, 25,* 91-98. https://doi.org/10.1007/BF00042179

Ministry of Agriculture &Livestock Development (2022) Statistical Information on Nepalese Agriculture (2021/22). https://s3-ap-southeast-1.amazonaws.com/prod-gov-agriculture/server-assets/publication-1595229368881-0dc12.pdf

Murashige, T., & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant 15(3),* 473–497.

MOAD (20/2017). Statistical Information on Nepalese Agriculture.

Nasib, A. S. M. A., Ali, K. A. S. H. I. F., & Khan, S. (2008). An optimized and improved method for the in vitro propagation of kiwifruit (*Actinidia deliciosa*) using coconut water. *Pak. J. Bot*, *40(6),* 2355-2360.

Negi, D., Saxena, S. (2010) Ascertaining clonal fidelity of tissue culture raised plants of *Bambusa balcooa* Roxb. using inter simple sequence repeat markers. New For 40(1):1–8. doi: 10.1007/s11056-009-9182-3.

Nookaraju, A., & Agrawal, D. C. (2012). Genetic homogeneity of in vitro raised plants of grapevine cv. Crimson Seedless revealed by ISSR and microsatellite markers. *South African Journal of Botany, 78*, 302-306.

Oliya, B. K., Chand, K., Thakuri, L. S., Baniya, M. K., Sah, A. K., & Pant, B. (2021a). Assessment of genetic stability of micropropagated plants of *Rhynchostylis retusa* (L.) using RAPD markers. *Scientia Horticulturae, 281,* 110008. https://doi.org/10.1016/j.scienta.2021.110008

Oliya, B. K., Kim, M. Y., & Lee, S. H. (2021b). In vitro propagation, lactucin quantification and antibacterial activity of Indian lettuce (*Lactuca indica L.). In Vitro Cellular & Developmental Biology-Plant,* 1-11.https://doi.org/10.1007/s11627-021-10234-9

Debenham, M., & Pathirana, R. (2021). Establishment and management of an in vitro repository of kiwifruit (Actinidia spp.) germplasm. In *Meta-Topolin: A Growth Regulator for Plant Biotechnology and Agriculture* (pp. 279-291). Singapore: Springer Singapore.

Qahtan, A. A., Faisal, M., Alatar, A. A., & Abdel-Salam, E. M. (2022). Callus-Mediated High-Frequency Plant Regeneration, Phytochemical Profiling, Antioxidant Activity and Genetic Stability in *Ruta chalepensis* L. *Plants, 11(12)*, 1614.

Rai, M.K., Phulwaria, M., Gupta, A.K., Shekhawat, N.S., Jaiswal, U. (2012) Genetic homogeneity of guava plants derived from somatic embryogenesis using SSR and ISSR markers. *Plant Cell Tiss Org Cult (PCTOC). 2012 Nov;111(2),* 259-64.

Rugini, E., Gutiérrez-Pesce, P., (2003) Micropropagation of Kiwifruit (*Actinidia spp*.). *In: Micropropagation of Woody Trees and Fruits* 647–669 https://doi.org/10.1007/978-94-010-0125-0_22

Saha, S., Adhikari, S., Dey, T., Ghosh, P. (2016 RAPD and ISSR-based evaluation of genetic stability of micropropagated plantlets of *Morus alba* L. variety S-1. *Meta Gene 7,* 7–15.

Sedaghathoor, S., Noie, M. (2016) Study on different grafting methods of kiwifruit 'Hayward'on the 'Matua'and 'Bruno'rootstocks. *EDP Sciences.*

Sharma, A., Thapa S., & Khatiwada, M. P.. 2020. "Production, Marketing and Future Prospects of Kiwifruit in Nepal." *International Journal of Applied Sciences and Biotechnology* 8 (2): 179–86.

Shen, X. S., Wan, J. Z., & Luo, W. Y. (1990). Propagation in vitro of Chinese Gooseberry (*Actinidia chinensis*) through the development of axillary buds. *Scientia Horticulturae*, *42*(1-2), 45-54.

Strik, B.C., Davis, A.J. (2021) *Growing Kiwifruit: A Guide to Kiwiberries and Fuzzy Kiwifruit for Pacific Northwest Producers*. In A Pacific North West Extension publication, Oregon State University.

Suezawa, K., Matsuta, N., Omura, M., & Yamaki, S. (1988). Plantlet formation from cell suspensions of kiwifruit (*Actinidia chinensis* Planch. var. chinensis). *Scientia horticulturae*, *37*(1-2), 123-128.

Sultana, K. W., Das, S., Chandra, I., & Roy, A. (2022). Efficient micropropagation of *Thunbergia coccinea* Wall. and genetic homogeneity assessment through RAPD and ISSR markers. *Scientific Reports*, *12*(1), 1683.

Thakur, J., Dwivedi, M. D., Sourabh, P., Uniyal, P. L., & Pandey, A. K. (2016). Genetic homogeneity revealed using SCoT, ISSR and RAPD markers in micropropagated *Pittosporum eriocarpum* Royle-an endemic and endangered medicinal plant. *PloS one*, *11*(7), e0159050.

Thapa, D.B., Karki, S. (2020) *Kiwifal kheti prawidhi pustika (Kiwifruit farming technology hand-book)*. National Fruit Development Centre, Ministry of Agriculture and Livestock. https://ncfd.gov.np/sites/default/files/2020-08/Kibi book-cover.pdf

Thomas, J., Vijayan, D., Joshi, S. D., Lopez, S. J., & Kumar, R. R. (2006). Genetic integrity of somaclonal variants in tea (*Camellia sinensis* (L.) O Kuntze) as revealed by inter simple sequence repeats. *Journal of Biotechnology*, *123*(2), 149-154.

Emaldi, U., Trujillo, I., & De Garcia, E. (2004). Comparison of characteristics of bananas (Musa sp.) from the somaclone CIEN BTA-03 and its parental clone Williams. *Fruits*, *59*(4), 257-263.

Vaidya, D., Vaidya, M., & Sharma, P. C. (2006, February). Development of value-added products from kiwifruit in India. In *VI International Symposium on Kiwifruit 753* (pp. 809-816).

Wang, T., Gleave, A. P. (2016) Applications of Biotechnology in Kiwifruit (*Actinidia*). In*: Innovations in Biotechnology (Issue tourism, p. 13)* Intech Open. https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics

Wang, T., Ran, Y., Atkinson, R. G., Gleave, A. P., & Cohen, D. (2006). Transformation of *Actinidia eriantha*: a potential species for functional genomics studies in Actinidia. *Plant cell reports*, *25*, 425-431. https://doi.org/10.1007/s00299-005-0080-7

Windauer, L. B., Insausti, P., Biganzoli, F., Benech-Arnold, R., & Izaguirre, M. M. (2016). Dormancy and germination responses of kiwifruit (*Actinidia deliciosa*) seeds to environmental cues. *Seed Science Research*, *26*(4), 342-350.https://doi.org/10.1017/S0960258516000192

Yue, J., Liu, J., Tang, W., Wu, Y. Q., Tang, X., Li, W., ... & Zheng, Y. (2020). Kiwifruit Genome Database (KGD): a comprehensive resource for kiwifruit genomics. *Horticulture research*, *7*.https://doi.org/10.1038/s41438-020-0338-9

Zhong, W., Zhou, J., Tang, D., Huang, Y., Liu, F., Zhang, M., ... & Tang, J. (2021). Establishment of tissue culture system of *Actinidia deliciosa* cultivar “Guichang”. *Journal of Chemistry*, *2021*, 1-9. https://doi.org/10.1155/2021/9951949

Tables and Figures of this article is presented in Annexure (Annex 2)

Annex 2

Table 6. Media detail used for *in vitro* callus regeneration and shoot tip culture of *Actinidia deliciosa* cv Alison

Media abbreviation	Basic medium	Growth hormones and additives			
		BAP (mg/L)	Kn (mg/L)	NAA (mg/L)	CW (%)
M1	FMS	0	0	0	0
M2	FMS	0.5	0	0	0
M3	FMS	1	0	0	0
M4	FMS	1.5	0	0	0
M5	FMS	2	0	0	0
M6	FMS	0.5	0	0.5	0
M7	FMS	1	0	0.5	0
M8	FMS	1.5	0	0.5	0
M9	FMS	2	0	0.5	0
M10	FMS	1.5	0	0.1	0
M11	FMS	1.5	0	0.2	0
M12	FMS	1.5	0	0.3	0
M13	FMS	1.5	0	0.4	0
M14	FMS	0	0	0	10
M15	FMS	0.5	0	0	10
M16	FMS	0	0.5	0	10
M17	FMS	0	0	0	15
M18	FMS	0.5	0	0	15
M19	FMS	0	0.5	0	15
M20	HMS	0	0	0	0
M21	HMS	0.5	0	0	0
M22	HMS	1	0	0	0
M23	HMS	1.5	0	0	0
M24	HMS	2	0	0	0
M25	HMS	0.5	0	0.5	0
M26	HMS	1	0	0.5	0
M27	HMS	1.5	0	0.5	0
M28	HMS	2	0	0.5	0
M29	HMS	1.5	0	0.1	0
M30	HMS	1.5	0	0.2	0
M31	HMS	1.5	0	0.3	0
M32	HMS	1.5	0	0.4	0
M33	HMS	0	0	0	10
M34	HMS	0.5	0	0	10
M35	HMS	0	0.5	0	10
M36	HMS	0	0	0	15
M37	HMS	0.5	0	0	15
M38	HMS	0	0.5	0	15

Table 2. Media detail used for *in vitro* rooting of *Actinidia deliciosa* cv Alison

Media abbreviation	Basic medium	Auxin used	
		IAA mg/L)	IBA (mg/L)
R0	FMS	0	0
R1		0.5	0
R2		1	0
R3		2	0
R4		3	0
R5		4	0
R6	HMS	0.5	0
R7		1	0
R8		2	0
R9		3	0
R10		4	0
R11	FMS	0	0.5
R12		0	1
R13		0	2
R14		0	3
R15		0	4
R16	HMS	0	0.5
R17		0	1
R18		0	2
R19		0	3
R20		0	4
R21		0	0

Table 3. *Acclimatization of* in vitro *rooted plants of* Actinidia deliciosa cv Alison *on different media*

SN	The medium used for acclimatization						
	Sand (V)	Soil (V)	Cocopeat (V)	Pearlite (V)	Vermicompost (V)	Carbendazim 50%	Trichoderma
AC1	1	0	0	0	0	1	0
AC2	3	1	0	0	0	1	0
AC3	1	3	0	0	0	1	0
AC4	1	1	1	½	0	1	0
AC5	1	1	1	0	0	1	0
AC6	0	2	1	½	0	1	0
AC7	0	3	1	½	0	1	0
AC 8	0	1	2	½	0	1	0
AC 9	1	1	3	½	0	1	0
AC10	1	1	3	0	0	1	0
AC11	1	1	3	½	1/2	1	0
AC12	0	1	3	0	0	0	1
AC13	1	1	0	0	0	1	0
AC14	1	1	0	0	0	0	1
AC15	1	1	0	0	0	0	0

Table 4. General characteristics of RAPD, ISSR and SSR primers used for evaluation of genetic fidelity in *Actinidia deliciosa* cv Hayward

	Name of primer	Sequence (5'-3')	Total band	Size range (bp)
RAPD	OPA-1	CAGGCCCTTC	3	600-1400
	OPA-3	AGTCAGCCAC	5	400-1500
	OPA-10	GTGATCGCAG	4	350-1000
	OPA-18	AGGTGACCGT	6	250-1500
	OPC-13	AAGCCTCGTC	2	300-1500
	OPC-20	ACTTCGCCAC	5	300-1500
Average			4.12	
ISSR	ISSR-874	AGAGAGAGAGAGAGAGTA	3	400-550
	ISSR-886	ACACACACACACACACGT	4	500-1500
	ISSR-889	AGAGAGAGAGAGAGAGCT	2	500-550
	ISSR-890	GACAGACAGACAGACA	6	430-1200
	UBC-812	GAGAGAGAGAGAGAGAA	3	430-700
	UBC -818	CACACACACACACACAG	4	290-1200
	UBC -827	ACACACACACACACACG	2	350-750
	UBC -825	ACACACACACACACACT	3	300-550
	UBC -834	AGAGAGAGAGAGAGAGYT	3	300-500
Average			3.33	
SSR	Ke115	F: AAGAAACAGGAGAGAGAATCG R:GGCACGTTGTACATCAGGAG	1	180
	Ke121	F: TCTGATTTCTCTCGCAGACG R: CATTTGTCCATCTGGCTGAA	1	350
	Ke129	F: CTGTGAAGATGGTGGGAAGC R: CAAATCAAGCCAATGACCAA	1	200
	Ke140	F: TCTTTCCCCTTCCCAAATCT R: TCTTGGGCTTGACAATCCAT	1	200
	Ke/sp	F: ATGTGAATCGATACGTGCGTG R: CTTAAGTTCTCGATTTAATCAG	1	200
	Ke217	F: AGCCCAGGGGAAACATCA R: GTGTGATCTGCACTCCCTGA	1	250
	Ke244	F: CGCCTTCCTGCTG R: CCCACCACCCAAA	1	340
Average			1	

Figures

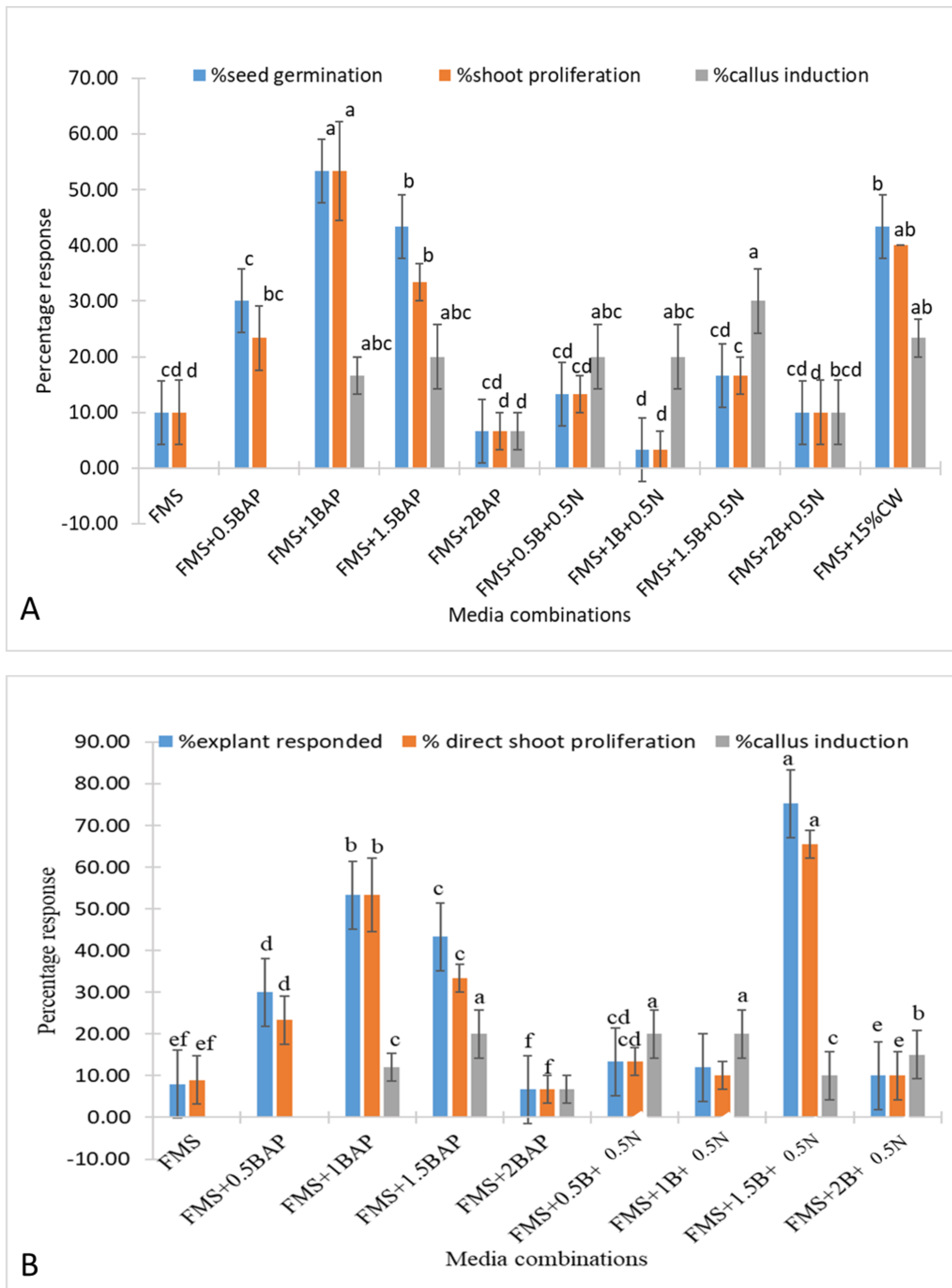

Fig. 1. *in vitro response of Actinidia deliciosa cv Hayward taken from field grown plant on FMS combined with various combinations of plant growth regulator. A: using seed as an explant, B: using shoot-tip as an explant. The bar graph represents Mean ± SE, and the different letters above the graph represent the significant difference between the mean value.*

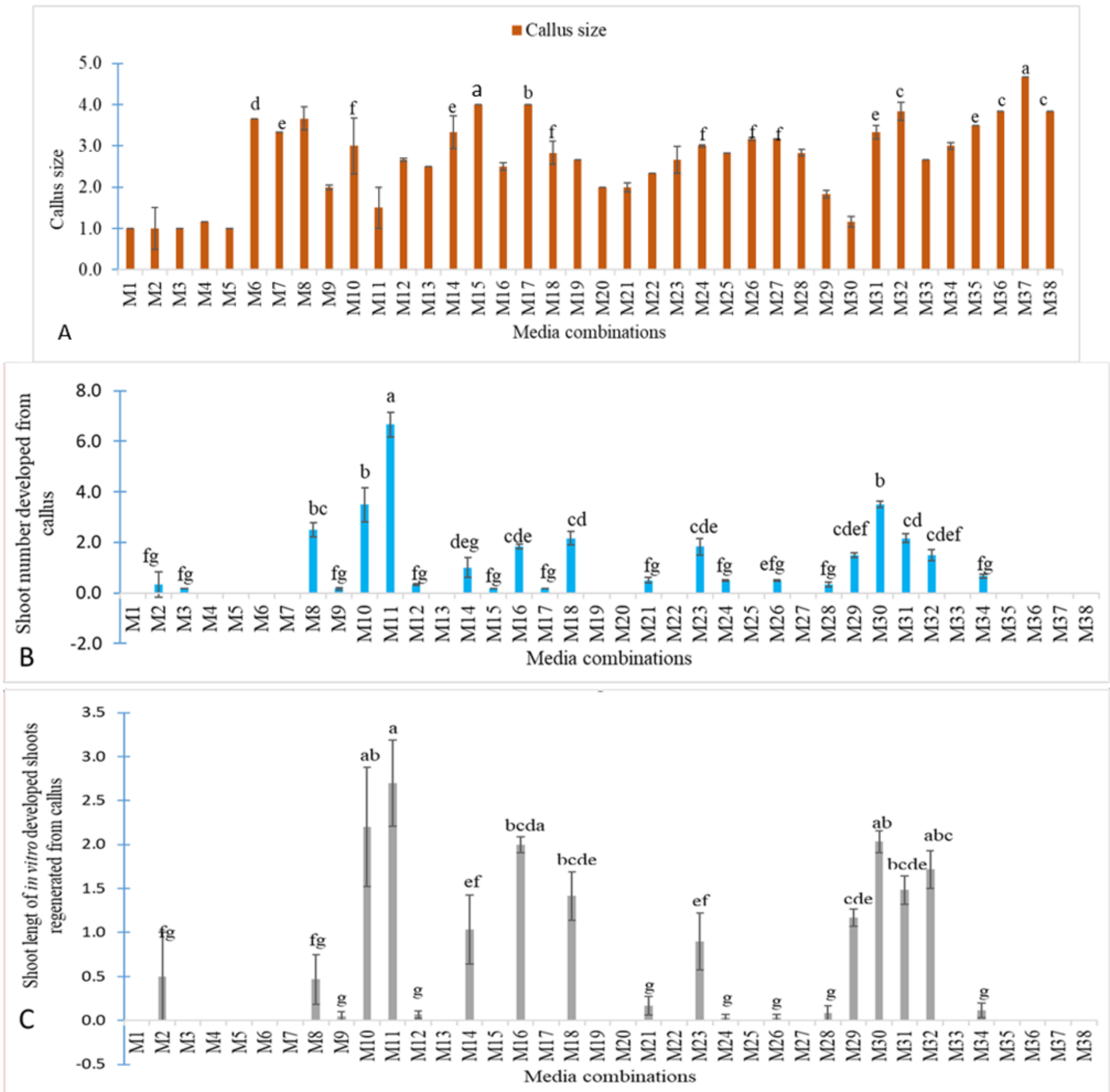

Fig. 2. *In vitro callus regeneration of Actinidia deliciosa cv Hayward in different media combinations. A: callus size development; B: shoot number formation; C: shoot length. For media abbreviation please refer to Table 1. The bar graph represents Mean ± SE, and the different letters above the graph represent the significant difference between the mean value.*

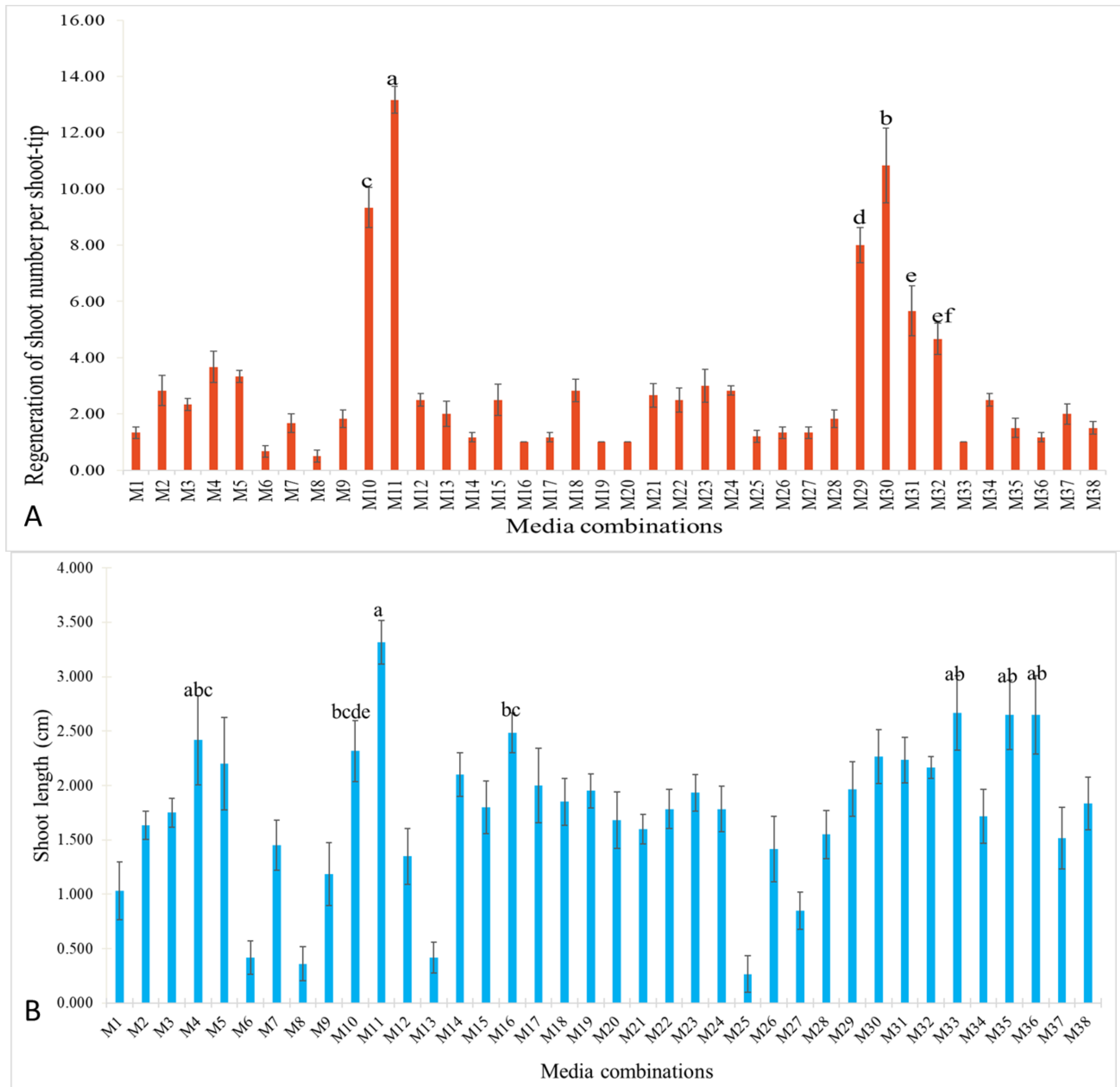

Fig. 3. *In vitro Shoot tip culture responses of Actinidia deliciosa cv Hayward for shoot number regeneration and shoot length formation. For media abbreviation please refer to Table 1. The bar graph represents Mean ± SE, and the different letters above the graph represent the significant different between the mean value.*

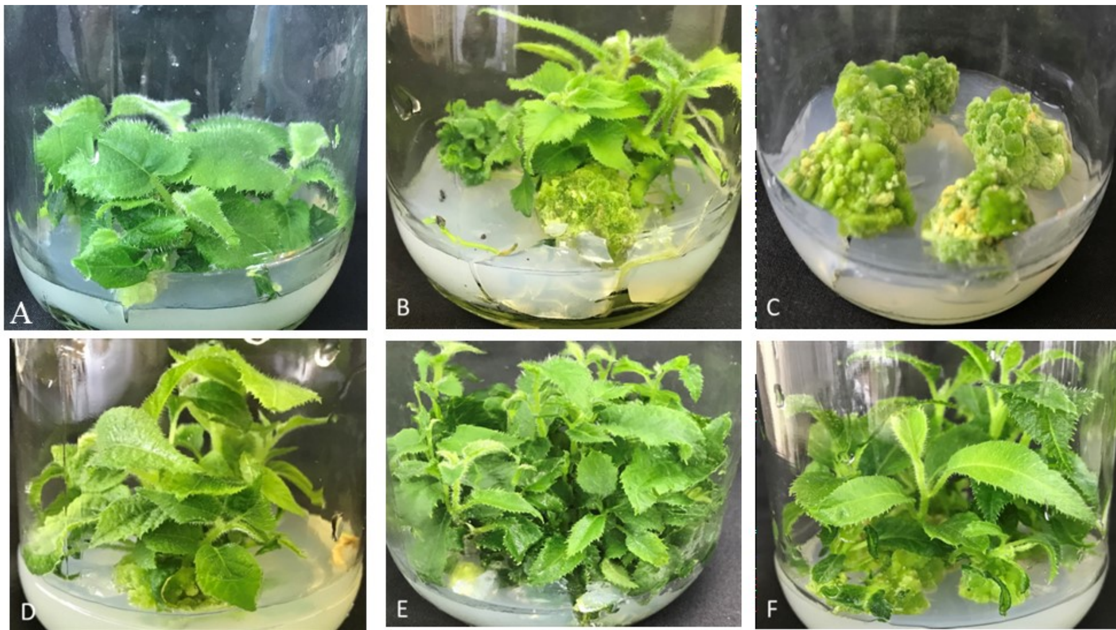

Fig. 4. *In vitro growth responses of Actinidia deliciosa cv Hayward. A: shoot-tip responded for shoot induction; B seed responses for callus development and direct shoot formation; C: callus enlarged in FMS combined with 15% CW; D: callus regeneration in FMS combined with 1.5 mg/L BAP + 0.2 mg/LNAA; E, F: shoot-tip and multiple shoot regeneration from shoot-tip and node culture on FMS combined with 1.5 mg/L BAP+0.2 mg/L NAA.*

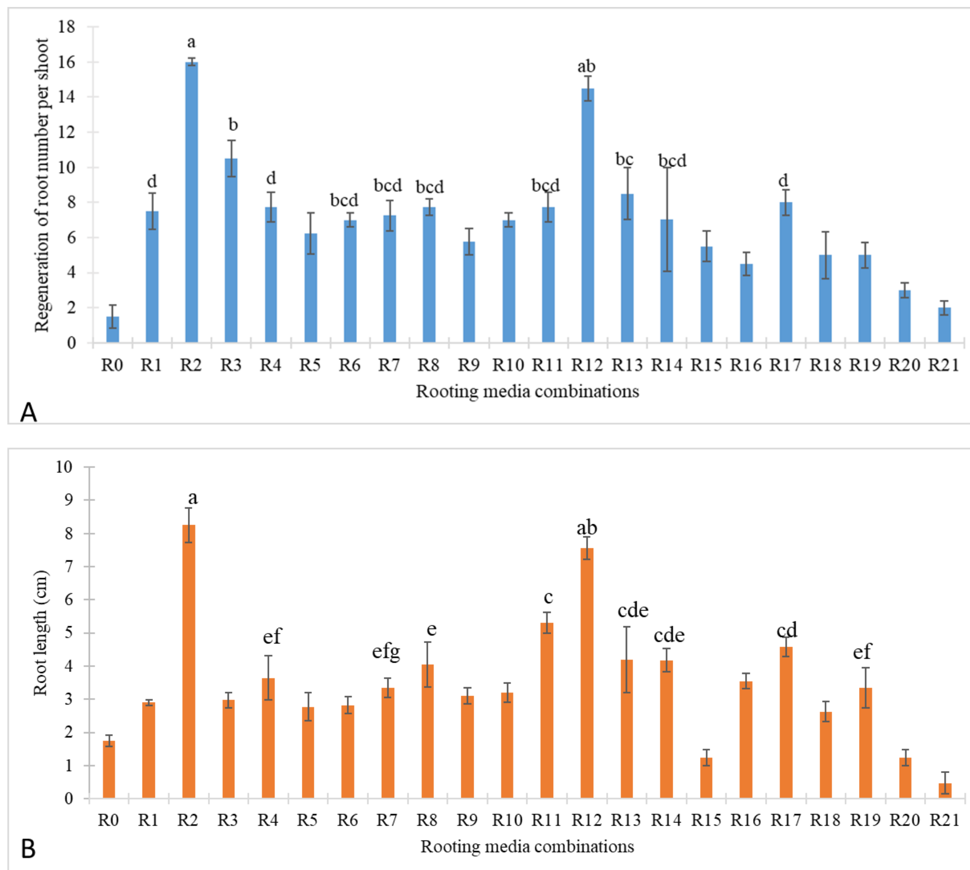

Fig. 5. *Responses of in vitro grown shoots of Actinidia deliciosa cv Hayward for in vitro rooting in FMS and HMS medium supplemented by various concentrations IAA and IBA. Media abbreviations used are- R0: FMS only; R1, R2, R3, R4, R5: FMS combined with 0.5, 1, 2, 3 and 4 mg/LIAA; R6, R7, R8, R9, R10: HMS combined with 0.5, 1, 2, 3 and 4 mg/l IAA; R11, R12, R13, R14, R15: FMS combined with 0.5, 1, 2, 3 and 4 mg/L IBA, R16.R17, R18,19,*

R20: HMS combined with 0.5, 1, 2, 3 and 4 mg/L IBA and R21: HMS only. The bar graph represents Mean ± SE, and the different letters above the graph represent the significant different between the mean value.

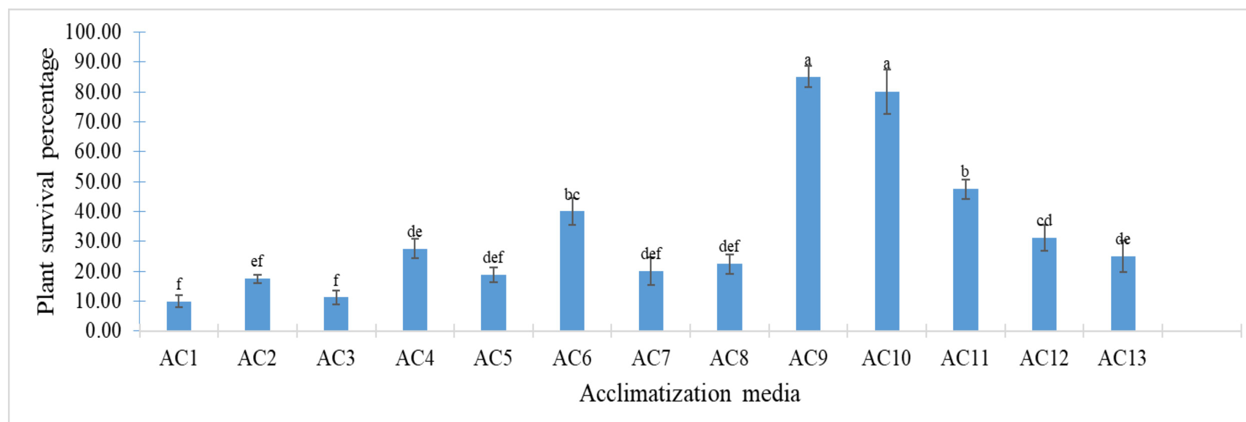

Fig. 6. Responses of in vitro grown shoots of *Actinidia deliciosa* cv Hayward for acclimatization. For acclimatization medium abbreviation please refer to Table 3. The bar graph represents Mean ±SE, and the different letters above the graph represent the significant different between the mean value.

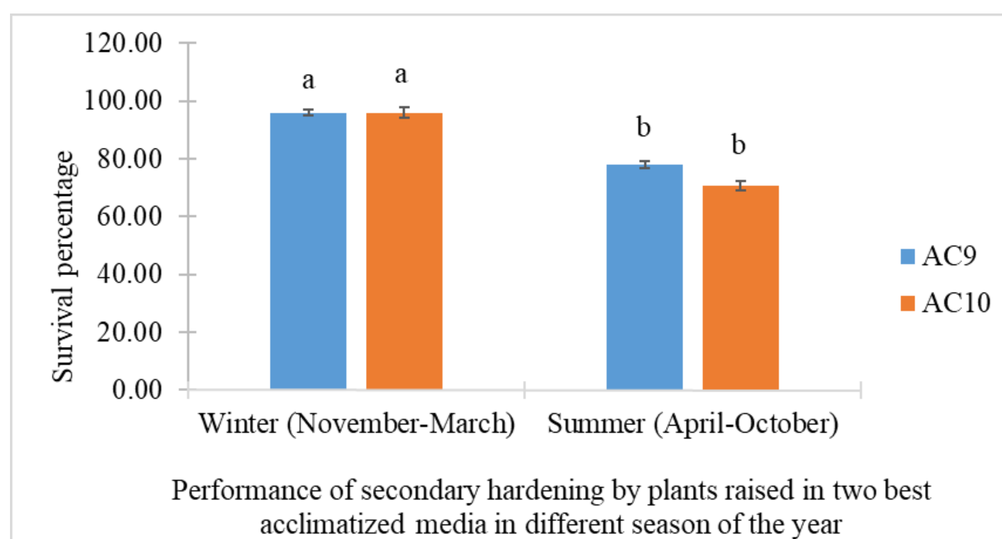

***Fig.** 7. Responses of primary hardened Actinidia deliciosa cv Hayward plants for secondary hardening during Winter (November-March) in glasshouse and Summer (April-October) in shednethouse Bar graph represents Mean ±SE, and the different letters above graph represent the significant difference between the mean value*

Fig. 8. *In vitro rooting and acclimatization of Actinidia deliciosa cv Hayward. A: rooting on FMS combined with 1mg/LIAA; B: rooting on FMS combined with 1mg/LIBA; C: well-rooted plant in primary hardening room; D: well-adopted primary hardened plants in a plastic cup at room temperature (30 days after transplantation to subtract at primary hardening room), E successfully survived plants at glasshouse during winter (after 1 month of transplantation from primary hardening to glasshouse); F: successfully survived plants at shed nethouse during summer (after 2 months of transplantation from primary hardening to shed nethouse)*

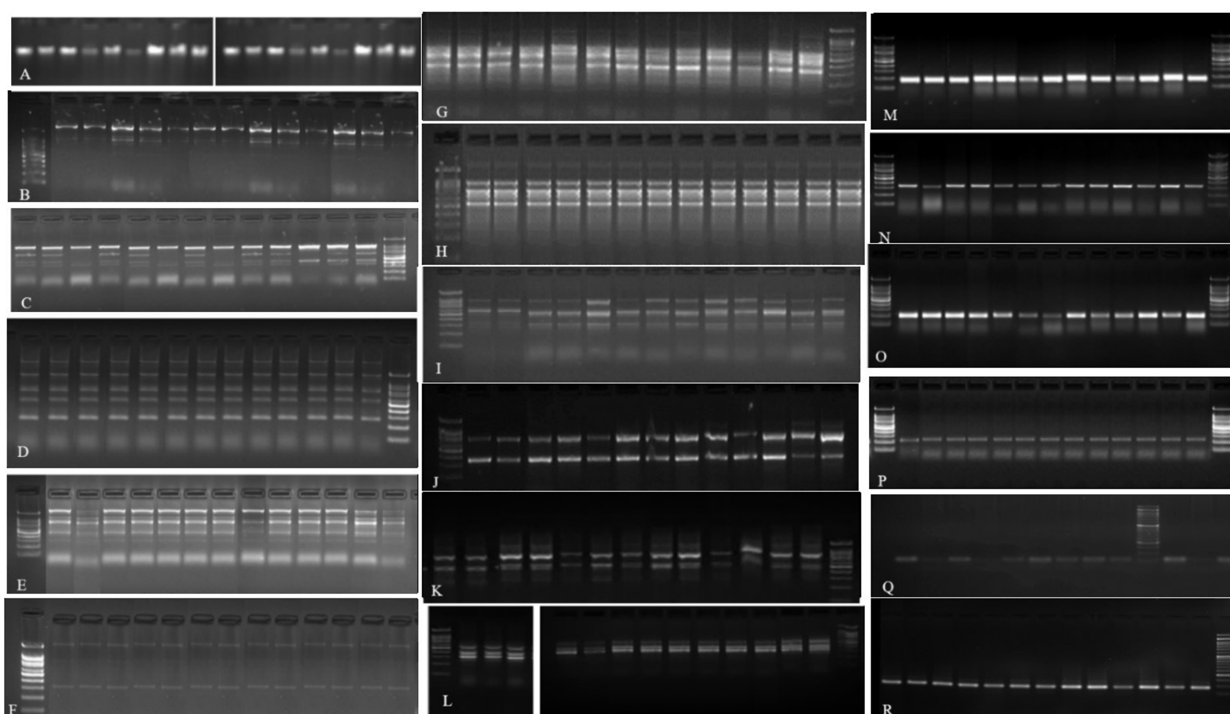

Fig. 8. *DNA marker based genetic evaluation of Kiwifruit Actinidia deliciosa cv Hayward. A: DNA image on 0.8% Agarose gel; RAPD markers B: OPA-1, C: OPA-6, D: OPA-10,E: OPA-18, F:OPB-4,G: OPC-13; ISSR markers G:ISSR 874, H: ISSR 886, I: UBC818, J: UBC 827, K:UBC 825 L: UBC 834. 1: mother plant (grafted Hayward), 2-13: in vitro plants developed from shoot-tip culture; L: 100 bp Ladder marker (CLS-MDNA-100bp DNA ladder RTU).*