

In vitro seed germination behaviour of different citrus rootstocks in MS basal media

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Abstract

An experiment was carried out during the year 2021-2023 at tissue culture laboratory, micro-propagation unit, Uttar Banga Krishi Viswavidyalaya, Pundibari, West Bengal to observe the seed germination behaviour and to standardize the seedling rootstock for *in vitro* shoot-tip grafting. The experiment was laid out in completely randomized design with three replications comprising eight different citrus rootstocks viz, NRCC 1, 2, 3, 4, rough lemon, rangpur lime, pummelo and mandarin (control) cultured in Murashige and Skoog basal media. Seeds were treated with Tween 80 for 15 minutes, followed by bavistin 500 mg for 1 hour, plantomycin 50 mg for 10 minutes and mercuric chloride 100 mg for 2 minutes before inoculation to remove its toxic residue from the seeds and also to avoid fungal and bacterial contamination. Seeds were thoroughly washed with double distilled water 3 times after each treatments. In each treatment, one hundred seeds were inoculated at five seeds per jam bottle. The results showed that rough lemon had the highest seed germination percentage (81.84%), followed by NRCC 4 (81.41%). The first sprouting of seed was observed in pummelo (5.49 days) followed by rangpur lime (5.59 days). The minimum days required for 50% of seed sprouting was observed in NRCC 4 (7.47) followed by mandarin (7.93). The minimum average days required for seed germination was observed in NRCC 3 (13.13) followed by NRCC 2. The germination index was found highest in NRCC 4 (59.00%) followed by NRCC 2 (44.16%). The polyembryony was observed highest in mandarin (24.19%) and rangpur lime (16.29%). The highest number of leaves was observed in rough lemon, pummelo and NRCC 4 (2.67). The rootstocks such as rough lemon, NRCC 4, pummelo and rangpur lime has sown potentiality for *in vitro* shoot-tip grafting.

Key words: mandarin, polyembryony, pummelo, *in vitro*, rangpur lime, rough lemon, shoot-tip grafting,

Introduction

Citrus is recognized as one of the most important perennial fruit crops in the world. India stands third highest citrus producer in the world after China and Brazil. Citrus is cultivated in more than 150 countries of which 53 countries cultivate it commercially. India contributes about 8% of citrus production in the world. In India, citrus is being cultivated in 1.10 million hectare with an annual production of 14.61 million metric tonnes (Anonymous, 2023). Citrus is evergreen in nature and belongs to rutaceae family which includes 130 genera and 1300 species distributed all over the world (Chhetri, 2019). Among the fruits, citrus is one of the most widely cultivated tropical and sub-tropical fruit due to its high nutritional value and plays vital role in industry sector. Besides being consumed as a fresh fruit, the processed products such as jam, marmalade, fruit juice etc. can be prepared. The fruit is very noteworthy because it is rich in organic minerals such as calcium, magnesium, potassium, copper, phosphorus, iron etc. and also contains vitamins such as thiamine, niacin, ascorbic acid, riboflavin, carbohydrates and fiber. The fruits has many phytochemicals and flavonoids compound such as anticancer, anti-microbial, anti-inflammatory and anti-oxidant properties which are essential for maintaining

normal growth and health. The fruits has a pleasant taste and aroma, which arises due to the presence of terpenes in the rind.

The fruit crops are perennial in nature and are prone to several biotic and abiotic stresses during their growth and development. Citrus trees are susceptible to many insect pest and diseases resulting in low yield, small sized and poor quality fruits. To tackle this problem rootstocks plays an important role because it influence various aspects of the overall plant, including growth rate, size, fruit quality, productivity, longevity of the tree, juvenility and tolerance to both biotic and abiotic stresses (Albrecht *et al.*, 2020; Gurung *et al.*, 2025). The rootstocks is considered as an essential component for successful citrus production in the modern age (Bowman *et al.*, 2016; Singh *et al.*, 2023; Singh *et al.*, 2024; Bey, 2025). By carefully selecting and using appropriate rootstocks, growers can optimize plant performance, improve yield, and enhance the overall health and resilience of the plants. A good citrus rootstocks should have a high degree of polyembryony, compatibility with the scion, adaptability to a particular soil stress (Shankar *et al.*, 2023; Vives-Peris *et al.*, 2023), tolerance to cold, drought, wind, and viruses like tristeza, exocortis, ringspot, fungal diseases, and nematodes, and also have a positive impact on the yield and

quality of fruits (Chand *et al.*, 2014), resulting in profitability of citrus production (Bowman *et al.*, 2016). Rootstocks such as rough lemon is vigorous in nature and have extensive root system and can be used for most of the citrus species but it is highly susceptible to *Phytophthora* which results in poor productivity. Rootstock such as Carrizo, Swingle, Sour orange, X-639, and Schaub showed tolerance to *Phytophthora nicotianae* (Singh, 2024). The potential to upgrade citrus rootstock is limited due to nucellar seedling, sexual incompatibility, apomixis, heterozygosity and ovule sterility (Mashaal *et al.*, 2024). The growth and vigour can be maintained by using dwarf citrus rootstocks for high density planting. It had been reported that the fruit colour in citrus is influenced by the rootstocks (Ladaniya, 2010). Seed propagation is the only preferred method for rootstock production in commercial citrus nurseries (Albrecht *et al.*, 2020). It involves careful management of seeds, seed storage period, germination condition, and knowledge of the requirements of seeds for overall growth and germination. Seed viability and germination percentage of some citrus cultivars remain high throughout the harvest season (Orbovic *et al.*, 2013). On the other hand, seed germination is a complex physiological process that mainly relies on the signals of environment such as temperature, moisture, humidity, light, etc. The speed of seed germination, maximum germination percentage, and healthy seedling stock are very essential to fulfilling the growing demands for budded and grafted-quality planting materials (Chand *et al.*, 2014). But there could be a chances of disease or pest transmission and infestation where the seedlings are grown in nursery or any open field. Micropropagation enables the bulking production of uniform disease free plantlets in a short period of time throughout the year, *in vitro* cloning of cultivated plants and different media combinations result in the maximum growth of citrus species (Mishra *et al.*, 2024). Therefore, the present study was planned to compare the germination attributes of different citrus rootstocks under *in vitro* condition. In citrus species, the technique of *in vitro* shoot-tip grafting has been reported to be very effective in eliminating viral diseases such as exocortis, xyloporosis, tristeza (Sanabam *et al.*, 2015). The demand of micrografted plants are very high in a citrus industry due to its ability to perform in different soil type, early fruiting, superior in quality, productivity and virus tolerant. But there is less fulfilment for the

supply of micrografted plants in citrus industry for the growers due to lack of knowledge about rootstocks attributes under *in vitro* condition. To fulfill the demands one must know about the germination behaviour of different rootstocks raised from *in vitro* conditions so that it could be easy to select the superior rootstocks for *in vitro* micrografting. It not only saves time but also minimize the extra expenses. In Kalimpong and Darjeeling regions many farmers were dependant on the Darjeeling mandarin because it is economically important and it plays an important role in the socio-economic upliftment of the peoples residing in this region made by selling mandarin but the area and production of mandarin in those regions has rapidly decreasing over the years due to an infection in an orchard by citrus tristeza virus, exocortis, xyloporosis and old senile orchard (Tarafder *et al.*, 2017). The old orchard can be replaced by the micrografted plants if the micrografted plants made available to the growers in those regions. For these *in vitro* production of rootstocks become an important steps to get the virus free plants. Keeping in mind these techniques, *in vitro* seed germination studies of eight different citrus rootstocks were carried out.

Materials and methods

The experiment was carried out at the plant tissue culture laboratory, micro-propagation unit under RKVY, Uttar Banga Krishi Viswavidyalaya, Pundibari, West Bengal during the year 2021-2023. The ripened fruits were collected from the seven year old healthy mother plants, from the orchard of Pomology and Post-Harvest Technology and the Regional Research Station, Kalimpong. One hundred seeds were previously subjected to running tap water for about 15 minutes using three drops of Tween-80 to remove excess dirt from the seeds. The seeds were later treated with fungicide (bavistin 500 mg/100 mL distilled water) for 1 hour followed by bactericides (plantomycin 50 mg/100ml distilled water) for 15 minutes to control bacterial and fungal infection. The seed inoculation was done inside the laminar air flow, seeds were sterilized with 100 mg mercuric chloride (HgCl₂) for 2 minutes, followed by 70 per cent (v/v) ethyl alcohol for 30 seconds, the seeds were thoroughly rinsed after completing every treatment with double sterile distilled water for three times to remove the toxic debris from the surface (Liberatore *et al.*, 2018), and

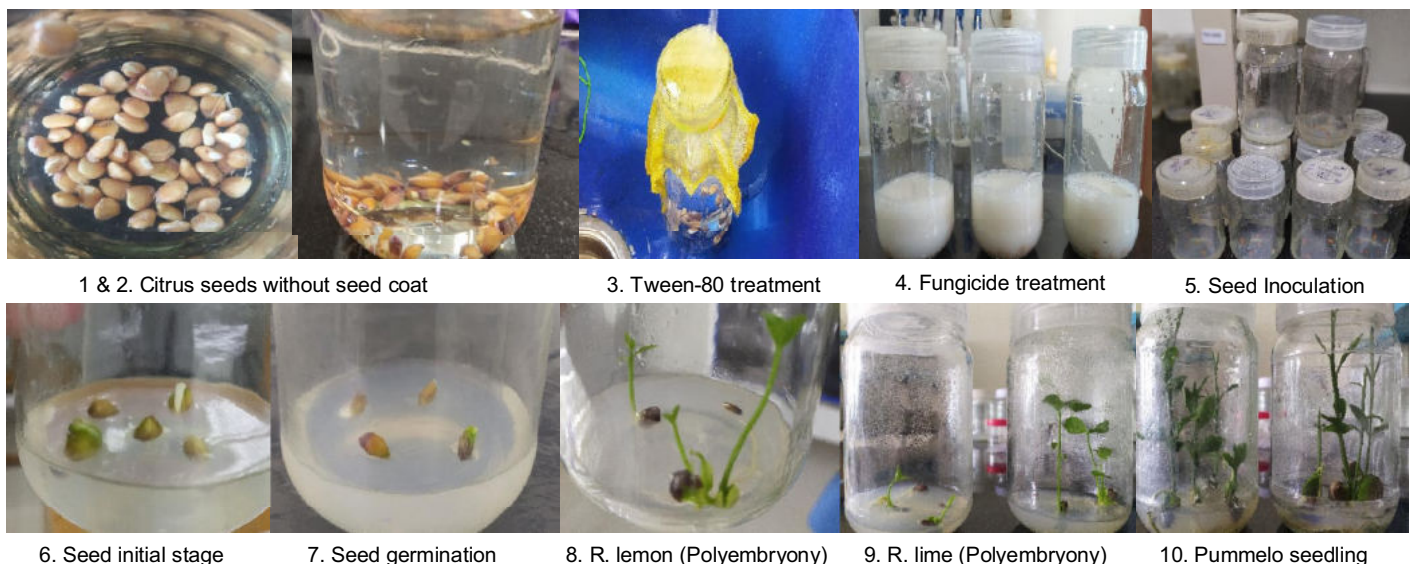


Fig. 1. Different steps in *in vitro* citrus seed germination

the seeds were transferred to another sterilized jam bottle for the seed inoculation. For seed germination, MS basal medium (Murashige and Skoog, 1962) fortified with 30g/L sucrose was used adjusting the pH to 5.8 before autoclaving. For the germination test, the treated seeds were cultured in jam bottle of standard MS basal medium containing sucrose and agar about 3% and 0.8%, respectively. All cultures were incubated under controlled condition at $24 \pm 2^\circ\text{C}$ temperature, $60 \pm 10\%$ relative humidity and a photoperiod of 14 hour with $20 \mu\text{mol m}^{-2}\text{S}^{-1}$ photosynthetic photon flux density (PPFD) provided by white fluorescent lamps (2×40 W, Phillips, India). The experiments was laid out in completely randomized design with 3 (three) replications comprising 8 (eight) different citrus rootstocks viz, NRCC 1, 2, 3 4, rough lemon, rangpur lime, pummelo and mandarin (control) were cultured in Murashige and Skoog basal media. In each treatment, 100 (one hundred) seeds were inoculated at 5 (five) seeds per jam bottle. The cultures were observed daily and the seed observation was made until the completion of the germination.

The seed germination percentage (G %) was calculated with formula using:

Days required for first sprouting: The seeds with 0.5 mm or more radical growth were counted as germinated seeds. When it observed for the first time in a seeds counted as a first sprouting.

The days required for 50% seed germination (T50) was calculated was calculated using (Coolbear *et al.*, 1984). As modified by (Farooq *et al.*, 2005).

$$D_{50} = \frac{t_i \left\{ \left(\frac{N}{2} \right) - n_i \right\} (t_i - t_j)}{n_j - n_i}$$

Where N is the final emergence, and n_j and n_i are the cumulative numbers of germinated seeds by adjacent counts at times, t_i and t_j respectively, when $n_i < N/2 < n_j$.

The average days required for seed germination or mean germination time (MGT) was calculated using the Ellis and Roberts equations (1981):

$$\text{MGT} = \frac{\sum (D * n)}{\sum n}$$

Where n is the number of seeds that germinates on D day, and D is the number of days counted from the starting of the germination time.

The Germination index (GI) was determined as described by the Association of Official Seed Analysis (1983) by the following formula:

$$\text{GI} = \frac{\text{No. of seeds germinated}}{\text{Days of first count}} + \dots + \frac{\text{No. of seed germinated}}{\text{Days of final count}}$$

Polyembryony (%): It was counted by visual observation of more than one embryos from the seeds during seedling emergence.

Shoot length (mm): The length of the shoots was measured from the basal part of the stem to the tip of the plants with measuring

tape after 14, 21 and 28 days of seed germination.

Root length (mm): The roots were separated from the media. Roots were further cleaned in running tap water and the media attached to roots were removed with the help of forceps. The length of the roots were measured from the growing point of roots to the longest available lateral root apex with the help of measuring tape after 14, 21 and 28 days of seed germination.

Number of leaves: After one month of germination, fifteen number of plants were selected from each treatment and counted by visual observation and average data were used.

Statistical analysis: All the data taken during the experiment were analyzed by separating mean using Duncan Multiple Range Test at $P=0.05$ (SAS Institute, Version 9.4).

Results and discussion

Among the eight different rootstocks seeds were placed in MS basal media, seed germination percentage was in the range of 70.08 to 81.84 %. However, seed germination percentage was recorded highest in rough lemon (81.84%) followed by NRCC 4 (81.41%) and NRCC 3 (80.93). The seed germination percentage of rough lemon are related with the findings obtained by Lahoty *et al.*, 2013; Sharma and Dhaliwal (2013); Ali and Mirza (2006); Singh *et al.*, 2015. The lowest seed germination was observed in NRCC 2 (70.08%) followed by NRCC 1 (77.67%) (Fig. 2). The first sprouting of all the eight rootstocks was in the range from 5.49 to 7.53 days. The minimum days required for first sprouting of seed was observed in pummelo (5.49) followed by rangpur lime (5.59). The maximum days required for first sprouting of seed was observed in mandarin (7.53 days) followed by NRCC 1 (6.58). Similar results was reported by Chand *et al.*, 2014. The minimum days required for 50% of seed germination was observed in NRCC 4 (7.47) followed by rangpur lime (8.75). The maximum days required for 50% of seed germination was observed in NRCC 3 (9.58) followed by NRCC 2 (9.56). The days required for 50% of seed germination was in the range of 7.47 to 11.00 days. The maximum average days for seed germination was recorded in mandarin (14.86) followed by rough lemon (14.15). The minimum average days for germination was recorded in NRCC 4 (11.78) followed by NRCC 3 (13.13). For seed germination, difference in time taken may be due to difference in genotype. Removal of seed coat before seed treatment reduce the time taken for germination (Hassanein and Azooz, 2003).

The germination index was in the range of 31.93 to 59.00. The germination index was recorded highest in NRCC 4 (59.00%) followed by NRCC 2 (44.16%). The minimum germination index was observed in mandarin (14.86%) followed by rangpur lime (31.93%). The highest polyembryony (%) was obtained from mandarin (24.19%) followed by rangpur lime (16.29%). It might be due to more number of nucellar embryos per seed. Nucellar embryos can be used to produce genetically uniform rootstocks to produce a variety with desirable and consistent characteristics. The similar results of polyembryony percentage was obtained by Singh *et al.*, 2015.

During the initial stage of vegetative growth of all the citrus rootstocks used for seed germination purpose, the shoot length was in between the range of 5.38 to 8.88 mm. there was an increasing development in shoot length till the four weeks after seed germination (Hassanein and Azooz, 2003). However, the

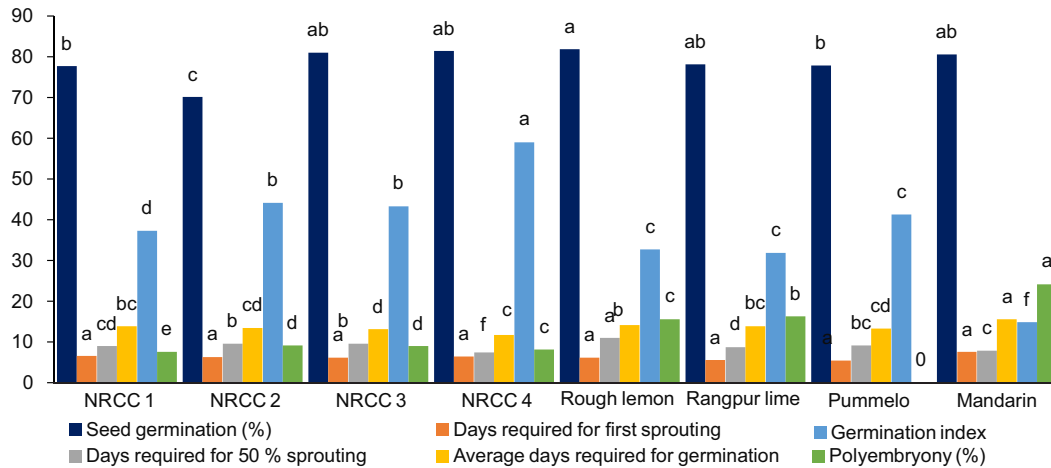


Fig. 2. Seed germination behaviour of citrus seeds in MS basal media.

maximum shoot length was recorded in pummelo (8.88 mm) followed by rangpur lime (7.35 mm) after two weeks of seed germination (Fig. 3). The minimum was observed in NRCC 4 (5.74 mm) followed by NRCC 2 (5.38 mm). After three weeks of seed germination it was again recorded highest in pummelo (15.69 mm) followed by rangpur lime (12.78 mm). But it was recorded lowest in NRCC 1 (9.31 mm) followed by NRCC 3 (10.14 mm). Again after four weeks of seed germination, the

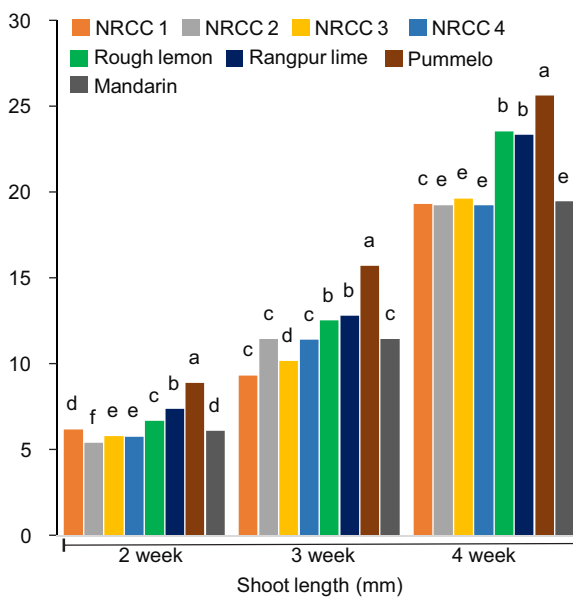


Fig. 3. Shoot length (mm) at 2nd, 3rd and 4th week

shoot length was recorded highest in pummelo (25.62 mm) followed by rough lemon (23.54 mm). The shoot length recorded for rough lemon is comparable with the result obtained by Naz *et al.*, 2007; Lahoty *et al.*, 2013; Chand *et al.*, 2014; Singh *et al.*, 2015; Chand *et al.*, 2014. The differences in shoot length of rootstocks might be due to their different growth habit. But it was found lowest in NRCC 4 (19.21 mm) followed by NRCC 2 (19.23 mm). After four weeks of seed germination shoot length was in between the range of 19.21 to 25.62 mm. The maximum root length was recorded in pummelo (4.30 mm) followed by rough lemon (3.27 mm) after two weeks of seed germination (Fig. 4). The lowest root length was observed in NRCC 3 (2.34 mm) followed by NRCC 4 (2.41 mm). Again after three weeks of seed germination, maximum root length was recorded in pummelo (5.81 mm) followed by Rangpur lime (3.79 mm). But it was found lowest in NRCC 1 (3.19 mm) followed by NRCC 4 (3.20 mm). After four weeks of seed germination, again the root length was recorded highest in pummelo (9.07 mm) followed by rangpur lime (6.79 mm). The lowest root length was observed in mandarin (6.05 mm) followed by NRCC 1 (6.14 mm). The root length was in between the range of 8.71 to 11.46 mm. The highest root length in pummelo is due to the superiority over other rootstocks. The highest number of leaves was recorded in pummelo, Rangpur lime, and NRCC 4 *i.e.*, (2.67). The lowest was recorded in NRCC 1, Rangpur lime and mandarin *i.e.*, (2.00). The root length of mandarin is similar with the findings of Chand *et al.*, 2014. However, the number of leaves obtained was in between the range of 2 to 2.67 in all the eight different rootstocks (Fig. 4). The length of shoot, root and number of

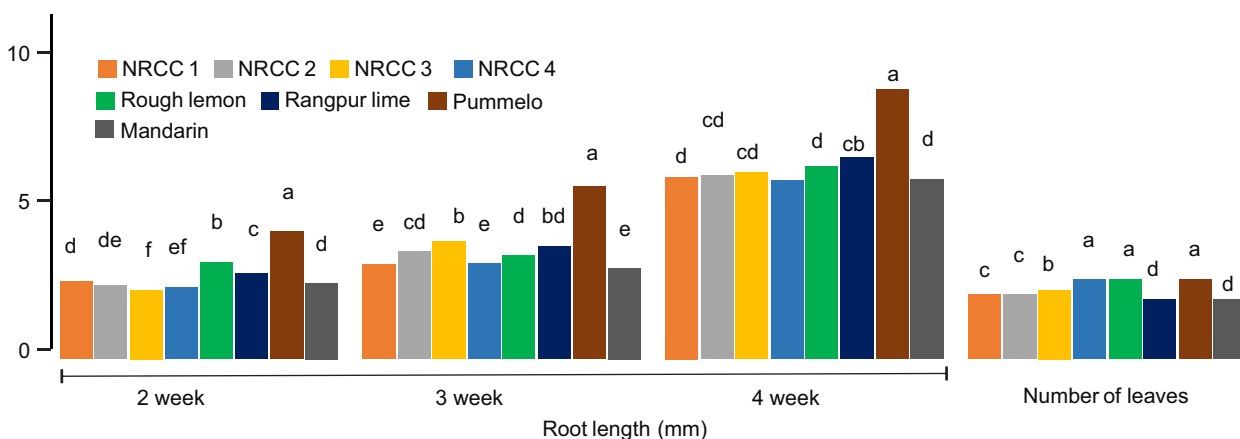


Fig. 4. Root length (mm) at 2nd, 3rd and 4th week and number of leaves in different varieties

leaves of both rough lemon and Rangpur lime are very with the findings of Singh *et al.*, 2015.

The study demonstrated that among the eight citrus rootstocks evaluated for seed germination behavior and initial vegetative growth, rough lemon, pummelo, and rangpur lime exhibited superior performance, showing the highest seed germination rates, the shortest germination periods, and the most robust vegetative growth. Polyembryony was observed in all rootstocks except pummelo under *in vitro* conditions. Previous studies have highlighted rough lemon and rangpur lime as having the highest success rates for micrografting and virus tolerance. Consequently, the findings of this research will assist scientists in efficiently selecting the most suitable rootstocks for micrografting in citrus under *in vitro* conditions, as these rootstocks outperform the others tested.

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