INTRODUCTION

The genus *Citrus*, one of the most important group of fruit crops worldwide, belongs to family Rutaceae comprising 140 genera and 1300 species distributed throughout the world. Citrus is believed to have originated in the part of Southeast Asia bordered by Northeastern India, Myanmar (Burma) and the Yunnan province of China (Gmitter and Hu, 1990; Scora, 1975). The well known examples of group citrus are oranges, lemons, grapefruits and limes. They are long-lived perennial crops grown in more than 100 countries across the world (Saunt, 1990). Favourable hotspots for citrus cultivation are tropical and sub-tropical areas, falling approximately within 400° latitude on each side of the equator, where temperatures are predominantly warm. The major citrus producing countries include Brazil, US, Spain, Italy, Egypt, India, Mexico and China. In India, citrus fruits rank third in area and production after banana and mango (Ghosh, 1997).

Citrus fruits are known for their distinctly pleasant aroma, arising due to terpenes present in the rind. The genus derives its commercial importance from its fruits, which are of great economic and health value and are consumed fresh or pressed to obtain juice (Talon and Gmitter Jr., 2008). Citrus peels too have no less importance and can be candied, used as livestock feed, in perfumeries, bakeries and in soap industry. Essential oils obtained from citrus leaves have recently been found to harbour insecticidal property. Lemon oil obtained by cold pressing of lemon peels is extensively used in furniture polish. Bergamot, a variety of sour orange is used in making perfumes and massage oils. The rind of citrus fruits is slightly bitter in taste and can be added to baked products to impart a distinct flavour. Citrus has been utilized in a number of medicinal preparations for the remedy of scores of ailments ranging from toothache, diarrhoea, constipation, insomnia to vomiting (Singh and Rajam, 2010).

The primary reason for shifting citriculture from seedling to budded plants was the appearance of *Phytophthora* “foot rot” in Azores Islands in 1842 (Singh and Naqvi, 2001). As the disease was recognized, the interest in root-stocks greatly increased because of heavy losses experienced among the susceptible seedlings. Thereafter, the
search for resistant root-stocks started and the seedlings were gradually replaced so that now-a-days nearly all the citrus trees are propagated by budding on to root stock seedlings.

Now-a-days, Citrus species are almost universally propagated by budding on to seedling rootstocks. Since early 1950s extensive rootstock trials on citrus have been conducted under different environmental conditions (Bhattacharya and Dutta, 1952; Rangacharlu et al., 1958 and Singh, 1962). Further, the citrus root stock scenario in India has been reviewed by Agarwal (1982), Randhawa and Srivastava (1986), Patil (1987) and Chadha and Singh (1990). The best performing rootstocks included Sour orange (Citrus aurantium), rough lemon (Citrus jambhiri), cleopatra mandarin (Citrus reticulata), trifoliate orange (Poncirus trifoliata), citrange (Citrus sinensis x Poncirus trifoliata), rangpur mandarin lime, volkamer lemon etc. Sour orange was considered to be the major rootstock because of its tolerance to different soil conditions, cold and foot rot but after the appearance of the new citrus threat by Citrus tristeza virus (CTV), the dominant sour orange rootstock has been replaced by rough lemon rootstock which was tolerant to CTV.

Rough lemon (Citrus jambhiri Lush.) is native to Northeastern India. Locally in Punjab it is known as “Jatti Khatti”. It is probably a natural hybrid because of its high degree of polyembryony as compared with other lemon species. In Punjab and nearby states, rough lemon has been considered to be the most important rootstock for lemons, oranges, mandarins, grape fruits and kinnows because of its high vigour, well adaptation to warm-humid areas with deep sandy soils and resistance to Citrus tristeza virus. It produces high yielding trees with large fruits. However, the main disadvantage is its susceptibility to Phytophthora spp. which leads to major losses in an orchard if proper phytosanitary conditions are not followed. Phytophthora species have been shown to cause some serious soil borne diseases of citrus including damping off of seedlings in the seedbed, root and crown rot in nurseries, foot rot and brown rot of fruits. This necessitates the production of Phytophthora tolerant nursery stock of Citrus jambhiri for getting healthier citrus trees with large quantity and good quality of fruits.
The potential of conventional methods of improvement of citrus rootstocks is limited by biological factors such as heterozygosity, inbreeding depression, nucellar polyembryony and juvenility. Under such circumstances tissue culture techniques offer best possible alternative for improvement and inducing variations and selection of variants for different needs. Plant tissue culture provides reliable and economical method of maintaining pathogen free plants that allows rapid multiplication and international exchange of germplasm. Tissue culture and micropropagation protocols have been described for a number of Citrus species from different explant sources (Barlass and Skene, 1982; Duran-Vila et al., 1989; Raman et al., 1992; El-Morsy and Millet, 1996; Normah et al., 1997; Chakravarty and Goswami, 1999; Al-Khayari and Al-Baharany, 2001; Filho et al., 2001; Khawale and Singh, 2005; Usman et al., 2005; Ali and Mirza, 2006; Altaf et al., 2008; Altaf et al., 2009a,b; Khan et al., 2009; Laskar et al., 2009; Sharma et al., 2009; Pe´rez-Tornero et al., 2010; Singh and Rajam, 2009, 2010). However, only a meager data on the tissue culture of rough lemon is available (Altaf and Ahmad, 1997; Ali and Mirza, 2006; Altaf et al., 2008; Savita et al., 2010, 2011a,b).

In fruit crops, mutagenesis has already been used to induce many useful traits affecting plant size, blooming time, fruit colour, fruit ripening, self incompatibility and resistance to pathogens (Janick and Moore, 1975; Lacey, 1975; Decourtye, 1982; Donini, 1982; Lapins, 1983; Spina et al., 1991; Masuda et al., 1997; Sanada and Amano, 1998). Genetic variations can be induced either by specific treatments with physical or chemical mutagens or by tissue culture (Larkin, 1998; Van den Bulk, 1991; Phillips et al., 1994). Tissue culture has the potential for improving effectiveness of mutation induction in several aspects. The availability of tissue culture protocols for plant micropropagation and regeneration from cells and tissue for nearly all of the commercially important fruits (George, 1993; Herman, 2000) calls for a wider use of in vitro mutation induction. Spontaneous and induced mutations have always been a source of genetic variations and have often proved useful for fruit tree breeding. Ethyl methane sulfonate (EMS) enhances genetic variability which can enhance resistance (Pius et al., 1994; Venkatachalan and Jayabalan, 1996; Bhagwat and Duncan, 1998; Jabeen and Mirza, 2002; Svetleva and Crino, 2005; Luan et al., 2007). A lot of work
has been done to induce mutations artificially by EMS in crop plants. Seed mutagenesis has been used for induction of early flowering in spring rape (Thurling and Depittayanan, 1992), herbicide tolerance in soybean (Sebastian et al., 1989), male sterility in wheat (Maan and Williams, 1984) and cucumber (Robinson, 1978), increased pollen variability and fruit rot resistance in bell pepper (Ashok et al., 1995) as well as quantitative variations in different yield traits in *Avena sativa* L. (Krishnamurthy and Vasudevan, 1984). High efficiency of EMS for creating variability, phenotypic variations like potato shaped leaves, reduced fruit size and maximum disease resistance have been observed in tomato (Yudhvir, 1996).

During the last 3 decades, lot of work has been done on the use of tissue culture methods for selecting the disease resistant plants against different pathogens. These studies have used cell free culture filtrates (CF) or pure toxins of the pathogen or even direct infection by the pathogen for the selection of disease resistance in plants (Hammond-Kosack and Jones, 1997; El-Kazzaz and Ashour, 2004). The *in vitro* selection and screening of plant cells or tissues resistant to fungal pathogens can be accomplished by using different screening agents such as purified specific or nonspecific toxins, crude extracts of pathogen cultures or by coculture with the fungus itself (El-Kazzaz and El-Mougy, 2007; Quaglia and Zazzerini, 2007; Kumar et al., 2008; Savita et al., 2011a). Induction of mutations for resistance to various pathogens at the cellular level and regeneration of plants may facilitate the selection of disease-resistant plants.

DNA markers have been applied to characterize variations (Somaclonal and induced) produced during *in vitro* conditions as they provide large number of reliable genetic markers for cultivars fingerprinting. RAPD (randomly amplified polymorphic DNA) analysis is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al., 1990). In citrus, PCR-based markers have been used for genetic mapping (Cai et al., 1994; Fang and Roose, 1995) to study genetic relationship among species or cultivars (Luro et al., 1992; Omura et al., 1993; Machado et al., 1996; Federici et al., 1998) to
discriminate citrus hybrids (Elísáriio et al., 1999) and to identify citrus mutants and periclinal chimeras (Deng et al., 1995; Sugawara et al., 1995).

Considering the susceptibility of Citrus jambhiri to Phytophthora and the potential of in vitro mutagenesis to produce stable genetic variants, the present study was planned to develop protocols for raising Phytophthora resistant/tolerant rootstocks of C. jambhiri (Jatti Khatti) employing in vitro mutagenesis. The main objectives of the present investigation include

- Survey and selection of healthy and diseased plants of Citrus jambhiri from the existing populations and their utilization as mother plants for in vitro studies.
- Establishment of protocols for raising aseptic cultures from various explants (nodal, leaf and root segments excised from in vitro raised seedlings; cotyledons, nucellar tissues, stigmas, styles, ovaries and juice vesicles).
- Raising of Phytophthora resistant/tolerant somaclonal lines of C. jambhiri through callus selection using explants from the Phytophthora infected plants.
- Induction of variations for resistance/tolerance to Phytophthora in the cultures of C. jambhiri employing chemical mutagen, ethyl methane sulfonate (EMS).
- Regeneration of Phytophthora resistant/tolerant callus lines of C. jambhiri (spontaneous and induced).
- Characterization of variations (somaclonal and induced) using RAPD Markers.
- Hardening, acclimatization and transplantation of in vitro raised plantlets to the field conditions.
- Field evaluation for the Phytophthora resistant/tolerant plants and their health monitoring.