Discussion
DISCUSSION

The current study reports for the first time wherein a low-cost micropropagation technology has been developed for an endangered medicinal herb endemic to North-Western Himalayas of India. The low-cost micropropagation technology has certain distinctive features over the previous reports of low-cost tissue culture alternatives in different plant species. For example, the technology has not only replaced some components of the nutrient medium with low-cost alternatives as reported in previous studies (Kaur et al. 2005; Patra et al. 1998), but also have worked out a complete process outline which depicts the amount of nutrient medium required for producing a defined number of *P. kurroa* plants in a particular period of time. This analysis would be of great practical use in setting up an industrial plant tissue culture unit not only for micropropagation of *P. kurroa* but also other plant species. More importantly the tissue culture process described in this study has rectified the problem of poor or low survival of tissue cultured plants of *P. kurroa* in the field conditions as reported in previous studies (Upadhyay et al. 1989; Lal et al. 1988). The identification of MS medium supplemented with KN (3mg/l) + IBA (2mg/l) for better shoot multiplication in *P. kurroa* is not very different from previous studies by Lal et al. (1988), who have identified kinetin with another auxin (IAA) as the best medium combination for *in vitro* shoot multiplication. Higher amounts of kinetin and IBA were used in the current study compared to previous studies, which can be attributed to the differences in genotypes of *P. kurroa* used. Another important refinement in the micropropagation of *P. kurroa* in the current study was that there was absolutely no occurrence of vitrified and fasciated shoots, which were reported in previous studies on tissue culture of *P. kurroa* (Upadhyay et al. 1989). The modifications in MS media by changing the concentrations and combinations of auxins and cytokinins did not help in obtaining shoots with good growth and well developed leaves. Since *P. kurroa* grows at high altitudes where temperatures are low, the incubation of auxiliary shoot tip cultures at low temperatures (15±1°C) helped significantly to provide better shoot growth with well developed leaves. The low-cost medium composition identified in the current study holds great promise in not only rapid multiplication of *P. kurroa* for its reclamation in natural habitat but also in rapid...
multiplication of genetically superior high phytopharmaceutical content strains of *P. kurroa* at a relatively lower cost compared to what has been reported so far.

The second component of study was identification of a suitable explant and medium composition for induction of callus cultures with the objective of seeing the feasibility of callus/cell suspension cultures for the production of medicinal compounds as well as induction of somaclonal variants. There are reports in medicinal & aromatic plants wherein the metabolites of medicinal importance are biosynthesized and accumulate in different organs such as roots, leaves and shoots (Ramachandra Rao and Ravishankar 2002). Similarly, the accumulation of medicinal compounds do occur in roots, rhizomes and shoots of *P. kurroa* (Ansari et al. 1988). The *P. kurroa* has been declared as an endangered medicinal herb due to its heavy exploitation from the natural habitat by various pharmaceutical industries and other vendors dealing with the marketing of medicinal plants. The endangered status thus warrants that tissue culture conditions need to be standardized from different parts of the plant so that the cultures can be used for the production of metabolites under laboratory conditions as has been reported in various medicinal plants so as to relieve pressure from its natural habitat (Vanisree et al. 2004).

The nutrient media forms the main component for induction of callusing and plant regeneration in tissue cultures. The major differences in the response of different plant species and different explants to tissue culture conditions lies with the ratio of auxins to cytokinins (Skoog and Miller 1957). High frequency callusing was achieved from root explants as compared to leaf disc and nodal segments in *P. kurroa* by using different modifications of MS media. Best callus formation was achieved in root explants compared to leaf disc and nodal segments. Similar response of higher callus induction from leaf explants has been reported in other plant species such as *Cichorium intybus*, *Clematis gouriana* Roxb. and *Plumbago zeylanica* (Nandagopal et al. 2006; Raja Naika et al. 2007 and Rout et al. 1999). During the callus formation, some of the calli turned into excessive roots. The root forming calli have been reported in other plant species such as from root explants of wheat (Chauhan and Singh 1995). These callus cultures,
therefore, could not be carried forward for differentiation into shoots and roots. The occurrence of high frequency callusing from root and leaf segments of *P. kurroa* is highly desirable because most of the phytopharmaceuticals accumulate in roots and shoots of *P. kurroa*. Thus the root- and leaf- derived callus cultures could be readily used for preparing cell suspension cultures for use in large scale production of medicinally important secondary metabolites in bioreactor conditions or even for understanding the biology of metabolites biosynthesis and accumulation in roots of *P. kurroa* as has been reported in other medicinally important plant species (Smetanska 2008).

Shoots were regenerated from different explant-derived calli in regeneration media containing different concentrations and combinations of auxins and cytokinins. Highest regeneration of shoots was observed from callus cultures-derived from root and leaf segments as has been reported in other plant species (Chang et al. 1980). The differential response of explants to callusing and regeneration may be due to varying concentrations of endogenous levels of auxins and cytokinins in different explants (Rout and Das 1997; Saxena et al. 1997; Patra et al. 1998; Jiménez et al. 2005). Rooted shoots were further hardened and transferred to field, hence a complete protocol for high frequency callus induction and plantlet regeneration has been standardized for *P. kurroa*. The protocol is expected to be of immense practical importance in selection and regeneration of cell lines of *P. kurroa* with enhanced content of medicinally important phytopharmaceuticals. The conditions of callusing and regeneration can also be used in genetic transformation of *P. kurroa* for increased phytopharmaceuticals production through metabolic engineering by using *Agrobacterium* -mediated transformation. Being endangered in its natural habitat, the cell/callus cultures can be cryopreserved for its sustainable conservation (Grout 2007). Moreover, the endangered status of *P. kurroa* also warrants taking up means for the production of phytopharmaceuticals through different approaches such as production of hairy root cultures (Verma et al. 2007) or elicitation of cell suspension or callusing cultures with biotic or abiotic elicitors for enhanced production of phytopharmaceuticals.
Plant tissue culture is a valuable technology for studying the biosynthesis and accumulation of secondary metabolites due to its inherent advantages over the conventional cultivation methods such as ability to produce useful compounds under controlled conditions. In addition, specific cells/tissue types of a particular plant species can be multiplied to produce higher yields of specific metabolites, which cannot be done through conventional methods of cultivation where programming of particular cells/tissues resulting in the production of specific metabolites becomes impractical. Plant cell culture technologies have made possible the production of a wide variety of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, monoterpens, flavonoids and amino acids, which are potential renewable resources for the production of valuable medicinal compounds, flavors, fragrances, pigments, dyes, cosmetics and fine chemicals (Matkowski 2008). Various factors such as physical, chemical, nutritional and genetic influence the production of metabolites in plant cell cultures. The commercial importance of secondary metabolites and the possibilities of their production by means of cell culture technologies have gained great interest in the recent years (Srivastava and Srivastava 2007; Namdeo 2007). For maximizing the production and accumulation of secondary metabolites through plant cell cultures, specific physical conditions such as type and composition of nutrient media, type and source of explant for initiating cell cultures, incubation temperatures and intensity of light, etc. are of paramount importance.

The current study is first of its kind wherein tissue culture conditions have been optimized for in vitro production of a medicinally important secondary metabolite, Picroside-1 of *P. kurroa*. A suitable explant and a nutrient medium composition have been identified for establishing shoot cultures of *P. kurroa*, which accumulate Picroside-1 to a higher level (2.0 mg/g fresh shoot wt.). The callus or root cultures of *P. kurroa* showed negligible amounts of Picroside-1 and Picroside-II. The biosynthesis and accumulation of medicinally important secondary metabolites has been reported to occur in developmentally different organs and tissues (Campos-Tamayo et al. 2008). It has been found that a certain level of cell differentiation is considered to be important in the successful production of phytochemicals by cell cultures (Song et al. 2007). In vitro production of saponins was carried out in callus and shoot cultures of *Gypsophila*
*Panax ginseng* grown in MS media supplemented with auxins and cytokinins (Hanafy et al. 2007). Whereas artemisinin production was carried out in shoot cultures of *Artemisia annua* (Liu et al. 2007). The hairy roots were found to accumulate kutkoside/picroside in root cultures of *P. kurroa* (Verma et al. 2007). The main constituents responsible for medicinal properties of *P. kurroa* are Picroside-1 and Picroside-II, however nothing is known about factors influencing their biosynthesis and accumulation, which are a prerequisite to go for either their *in vitro* production or for genetic manipulation of *P. kurroa* for their enhanced production. The biosynthesis and accumulation of Picroside-1 and Picroside-II has been reported to occur differentially wherein the Picroside-1 accumulated preferentially in leaves, whereas roots contained higher amount of Picroside-II (Dutt et al. 2004).

In most of the tissue culture studies on various plant species the cultures were incubated at 25±1°C, including published reports in *P. kurroa* (Lal et al. 1988). The shoots of *P. kurroa* formed at 25±1°C were thin and slender with narrow leaves, low yield of shoot biomass and the plants could not be successfully established under field conditions. Testing of different modifications of basal MS medium with different combinations and concentrations of growth hormones (auxins and cytokinins) did not make any difference in obtaining profuse growth of shoots with well developed leaves when cultures were incubated at 25±1°C. Moreover the biosynthesis and accumulation of Picroside-1 and Picroside-II were negligible in shoots or roots formed at 25±1°C.

The incubation of *P. kurroa* shoot cultures at different temperatures revealed that the total shoot biomass yield per shoot was significantly high (3.73g) in shoots formed at 15±1°C compared to incubation of shoot cultures at other three temperatures (10±1°C, 25±1°C, 30±1°C). The incubation of shoot cultures at 15±1°C also showed significant differences for leaf size and shoot biomass yield compared to incubation of shoot cultures at other temperatures. The accumulation of Picroside-1 was also high (2mg/g fresh shoot wt.) in shoots formed at 15±1°C after 4 weeks of incubation compared to shoots formed at other test temperatures. Though the accumulation of Picroside-1 occurred in shoots cultured at 10±1°C yet the growth and multiplication of shoots was low compared to
other incubation temperatures. Thus the incubation of *P. kurroa* shoot cultures at 15±1ºC was found to be optimal for obtaining higher biomass yield coupled with their enrichment for medicinally important metabolite, Picroside-1.

Plants tissue cultures are largely influenced by the quality and duration of light and temperature treatments. Heat shocks of 35-50ºC for 30-60 min in the suspension cultures of *Taxus yunnanensis* were used for enhancing the production of paclitaxel (Zhang et al. 2007). The production of swertiamarin and gentipicroside was enhanced in cell suspension cultures of *Gentiana davidii* by incubating at 25ºC and light intensity of 2.33 Lux (Chueh et al. 2000). Testing of different temperature regimes i.e. 15, 20, 25, 30 and 35ºC on different germplasm lines of *Hypericum perforatum* revealed that levels of hypericin, pseudohypericin and hyperforin were increased in shoots concomitant with the increased leaf total peroxidase activity at 35ºC (Zobayed et al. 2005). Dark-grown suspension cultures of *P. hexandrum* accumulated 0.1% podophyllotoxin, which was three- to fourfold that in light-grown cultures (Van Uden et al.1993). The effects of temperature shift on cell growth and paclitaxel production in suspension culture of *T. chinensis* was studied. Cell growth was optimum at 24ºC, while paclitaxel synthesis reached a maximum at 29ºC (Choi et al. 2000).

The observation that the MS medium containing a particular combination of cytokinin and auxin i.e. KN (3mg/l) + IBA (2mg/l) + agar-agar (8.0g/l) + sucrose + (30g/l) was suitable for better growth and multiplication of shoots as well as maximum accumulation of Picroside-1, implied that the profuse growth and development of *P. kurroa* shoots *in vitro* is concomitant with the biosynthesis and accumulation of Picroside-1. Concentrations and combinations of growth hormones i.e. auxins and cytokinins have been found to influence the biosynthesis and accumulation of secondary metabolites in different plant species. The best scopoletin levels were obtained with auxin concentrations such as 0.2 mg/l 2,4-D, 2 mg/l 2,4-D, 10 mg/l NAA, and 20 mg/l IAA in cell suspension cultures of *Angelica archangelica* (Siatka and Kasparová, 2008). Highest yield of berberine (4.14 times to that of control) was obtained with 4 mg/l of NAA, while the best cell growth (~ 2.00 times to that of control) was observed in the presence of 2
mg/l of 2,4-D (Narasimhan and Nair 2004). The production of gentipicroside and swertiamarin was enhanced on MS medium supplemented with kinetin, NAA and 3% sucrose in suspension cultures of *Gentiana davidii* (Chueh et al. 2000).

The production of medicinal compounds economically by extraction from intact plants and meet the ever-increasing demand is not a sustainable approach, particularly when high value medicinal plants are being declared as endangered. This may be due to very low concentrations of these active compounds in plants, the slow growth rate of plants, complex accumulation patterns, and high susceptibility to geographical and environmental conditions. Other possible reasons are the non-availability of uniform and unadulterated quality plant material in quantities sufficient for industrial production and uneconomical chemical synthesis, particularly for large complex molecules. Therefore, biotechnological methods offer an excellent alternative for production of such compounds. To overcome all these hurdles, the industry requires alternative methods of supply of uniform material throughout the year. Plant cell culture technology is undoubtedly one of the appropriate approaches to solve the above-described problems. The goal should, therefore, be to improve the productivity of cells in order to make the production of these compounds commercially viable. Commonly, no single growth-enhancing strategy can produce such a large increase, but in many cases the simultaneous application of multiple strategies results in synergistic interaction and thus an improvement of the yield.

There is widespread interest in the application of plant tissue culture methods and biotechnological approaches to the production of medicinal plants and isolation of medicinal secondary products. When compared to traditional agricultural growth, plant tissue culture production of medicinal plants offers a number of unique advantages, including the possibility of year-round, continuous production of plant medicinal compounds under highly controlled conditions. As the *in-vivo* production of secondary metabolites by plants can be highly influenced by plant growth environment factors such as climatic and soil conditions, pathogen attack and herbivory (Srivastava et al. 1995; Oksman-Caldentey and Inze 2004), *in-vitro* tissue culture growth of medicinal plants can
provide a route for consistent medicinal chemical isolation from plant materials (Tabata et al. 1991). Indeed, the lack of consistency in the levels of active chemicals in herbal medicines has been a continual issue (Bisaria and Panda 1991) and variation in secondary metabolite production in agriculturally grown plant material can certainly contribute to this problem. Plant tissue culture growth of medicinal plants can also be scaled up using continuous culture systems such as “bioreactors”, and this could allow for automated, high level isolation of medicinal secondary products (Yamada 1984; Benjamin et al. 1986). This would be particularly advantageous for secondary metabolite production from medicinal plants that are slow-growing in vivo, and could eliminate concerns regarding over-harvesting of medicinal plants which are either rare or endangered (Yamada 1984; Benjamin et al. 1986).

There was no change in the levels of Picroside-I and Picroside-II on different concentrations of sucrose in the medium. The manipulation of the amount and source of sugar has been found to enhance growth and secondary metabolite production (Stierle et al. 1993). Elevated sucrose levels were favorable in some cultures (Altstadt et al. 2001), and the addition of fructose promoted taxol production in Taxus spp. cell cultures (Pezzutto 1996).

The differential accumulation of Picroside-I content in shoots derived from callus cultures of leaf, nodal and root segments in our study may be due to the differences in growth and development of shoots derived from there explants. The shoots regenerated from root derived callus cultures were comparatively slow in growth and less developed. These observations indicate that the stage of growth and development in cell cultures is an important factor in the regulation of biosynthetic pathways. The biosynthesis and accumulation of secondary metabolites have been found to vary among plant parts and morphological stages as well as different morphogenetic tissue culture stages (Brown 1986). The ellagic acids present in Rubus chamaemorus plants was 3 times lower in shoot cultures and over 10 times in callus cultures (Thiem and Krawczyk 2003). Similarly in S. officinalis (Grzegorczyk et al. 2007) and Roseb rosemary (Caruso et al. 2000) in vitro cultures, the abietane diterpene antioxidants (carmisol and carnosic acid ) were present
only in shoot cultures and not in callus, suspension or hairy roots. Similar observations have been made in the biosynthesis of vindoline in Catharanthus roseus, wherein the metabolite does not accumulate in the callus cultures but occurs specifically in the shoots (St-Pierre et al. 1999; Constabel et al 1982; Aerts et al. 1992). The artimisinin production has been minimal in undifferentiated callus and cell suspension cultures of Artemisia annua (Liu et al. 2003). A certain degree of differentiation of shoot cultures was a prerequisite for artimisinin biosynthesis (Paniego and Giulietti 1996).

The lack of biosynthesis of Picroside-I in undifferentiated callus cultures of P. kurroa can be attributed to the absence of proper cell organization and programming of cell machinery involved in the biosynthesis of Picroside-I. The modification of relative biosynthesis to degradation ratios of a desired product can also influence the final levels of a desired compound in culture. However, whether biosynthesis of Picroside-I is shut down in callus cells or there is degradation of Picroside-I in callus cells of P. kurroa remain to be understood. The observation that biosynthesis and accumulation of Picroside-I occurs preferentially in differentiated shoots of P. kurroa and not in de-differentiated callus cultures, whereas there is no biosynthesis of Picroside-II neither in differentiated shoot/root cultures nor in de-differentiated callus cultures of P. kurroa opens up several avenues to investigate biology of Picroside biosynthesis in more detail. The Picroside-I and Picroside-II belong to sesquiterpene class of terpenoids (Negi et al 2008) and organogenesis has often been used as a means of inducing monoterpene production in tissue cultures, which otherwise fail to produce monoterpens as long as they are maintained in morphologically undifferentiated state, e.g. valepotriate production was increased by the induction of root morphogenesis in callus cultures of Valeriana officinalis and positive correlation was observed between the levels of differentiation and valepotriate production (Shrivastava et al. 2006). The results of current study indicate that the in vitro cultured shoots of P. kurroa possess biosynthetic potential for Picroside-I production, which can be exploited for exclusive production of Picroside-I on a large scale under proper growth conditions.
It has been observed that the callus/ cell suspension cultures used in *in vitro* production of phytochemicals are genetically unstable due to spontaneous occurrence of variant cell lines, which in turn results in the deterioration of metabolite yield with the passage of culture duration. The uniformity of Picroside-1 content in shoot cultures of *P. kurroa* after repeated sub-culturing for 6 months demonstrates genetic homogeneity of shoots over longer periods of culture. The production of plant metabolites through shoot cultures is thus advantageous from genetic uniformity perspective.

The recognition that certain secondary metabolites such as phytoalexins are produced by plants in response to microorganisms has led to the concept of using stimulators (known as elicitors) for enhancing production of phytochemicals in *in vitro* cultures (Namdeo et al. 2008). Enhanced production of many valuable secondary metabolites by using various elicitors have been reported successfully in various plant species (Wang and Zhong 2002; Dong and Zhong 2001; Hu et al. 2001; Yu et al. 2002). Among these, the yeast extract and methyl jasmonate have been found to be the most potent in stimulating the biosynthesis of terpenoids in plants (Szabo et al. 1999). On the same analogy the testing of yeast extract or MeJ on biosynthesis and accumulation of Picroside-1 in *P. kurroa* revealed that up to 4 fold increase in the Picroside-1 content was observed in the shoot cultures after the treatment of MeJ. Jasmonic acid (JA) and its methyl esters, methyljasmonate (MJ) have been reported as key signaling compounds in elicitation leading to enhanced accumulation of various secondary metabolites (Szabo et al. 1999). Higher saponin production (~28 folds) was found in the elicited cultures of *Panax ginseng* by using yeast extract & methyl jasmonate as elicitors (Lu et al. 2001). Dramatic increase in hypercin production was reported after elicitation of cell suspension cultures of *Hypericum perforatum* with Jasmonic acid (Walkar et al. 2002). The addition of methyl jasmonate to cell cultures of *Forsythia intermedia* resulted in three- and sevenfold accumulations of the lignans, pinoresinol and matairesinol, predominantly as glucosides (Schmitt and Petersen 2002). The effect of adding methyl jasmonate to various cell lines in suspension culture of *Linum album* has also been studied, with a twofold increase in podophyllotoxin (7.69±1.45 mg g⁻¹ dry weight) and 6-methoxypodophyllotoxin (1.11± 0.09 mg g⁻¹ dry weight) being achieved as maximum in
one of the cell lines (Van Furden et al. 2005) Enhanced production of podophylotoxin in suspension cultures of *Linum album* was reported by using biotic (Yeast extract) and abiotic (Ag⁺, Pb²⁺ and Cd²⁺) elicitors (Mohammadreza et al. 2005). Methyl jasmonate treatment led to large improvements in both cost and efficiency over the use of undefined fungal extracts, 100 pM methyl jasmonate increased the paclitaxel level from 28 to 110 mg L⁻¹ in a *T. media* cell line, and from 0.4 to 48 mg L⁻¹ in *T. baccata*. Other groups have reported similar effects of methyl jasmonate in cell cultures of *T. cuspidata* (Mirajilili and Linden 1996), *T. canadensis* and *T. cuspidata* (Ketchum et al. 1999) and *T. media* (Baebler et al. 2002). Significant enhancement of taxoid production by repeated elicitation using methyl jasmonate in bioreactor cell cultures of *T. chinensis* has also been reported.

Nineteen fold increase in the paclitaxel production was found in the elicited cultures of *Taxus cuspidate* by using methyl jasmonate along with ethylene as elicitors (Mirjalili and Linden 1996). Methy jasmonate, vanadyl sulphate and chitosan were used for enhancing the production of ginsenoside from hairy root cultures of *P. ginseng* (Palazon et al. 2003). The MeJ has also been reported to enhance the biosynthesis of various terpenoids such as artimisinin production in cell suspension cultures of *Artemisia annua* (Baldi and Dixit 2007), production of hypericin and hyperforin in *in vitro* cultures of *Hypericum perforatum* (Pavlić et al. 2007), and increased production of triterpenoids in *Centella asiatica* and *Galphimia glauca* (Mangas et al. 2006).

Mistaken or willful adulteration of medicinal plant material can be a major problem in a wide range of situations. These include, for example, the production of traditional Chinese medicine prescriptions that often consist of a complex mixture of ingredients, or the manufacture of certain herbal products, that are regarded as dietary supplements in Western countries and therefore not subjected to stringent safety controls. The problem is perhaps best exemplified by the major medicinal crop ginseng. Cultivated common ginseng *Panax ginseng* C. A. Mey. fetches only about 10–20% of the value accorded to *American ginseng Panax quinquefolius* L., and is therefore often marketed under the more “expensive” name. Even worse, completely unrelated and/or even
poisonous species from various genera with similar-looking roots (e.g., *Bletilla*, *Curcuma*, *Gymura*, *Mirabilis* and *Phytolacca*) are sometimes also marketed as ginseng.

The introduction of PCR-based molecular marker methods during the early 1990s constituted a new milestone in the field of DNA fingerprinting, two methods using single primers with arbitrary sequence were published in 1990 [Welsh and McClelland, 1990; Williams et al. 1990]. Numerous variants followed. These usually short, arbitrary primers were shown to generate anonymous PCR amplicons from genomic DNA, resulting in polymorphic banding patterns after gel electrophoresis and staining. The RAPD (random amplified polymorphic DNA) approach developed by Williams et al. [1990] has become the most popular variant of this “prototype” of PCR-based DNA profiling, mainly because of its technical simplicity. There DNA fingerprinting approach have the potential to be used as DNA based diagnostics for proper identification of adulterated and true to type plant material.

Breeders can generate genetic variability occurring in cell and tissue cultures *in vitro* for the genetic improvement of plant species. The induction of somaclonal variants for enhanced Picroside-I content has been found in the current study. Two of the somaclonal variants showed 1.5 to 2.0x fold increase in Picroside-I content over the parental genotype of *P. kurroa*. The RAPD analysis of somaclonal variants showed wide range of variations for presence or absence of amplicons for a particular locus, variations in number of bands, etc. Interestingly, two high Picroside-I content somaclonal variants showed common and RAPD fingerprinting patterns. Selection can be performed on the cell level either *in vitro* or on the level of fully functional plants regenerated from tissues or cells. Somaclonal variation has been observed for various agronomic traits in plants, such as great variability was found for total alkaloid production among protoplast-derived regenerants of *Hyoscyamus nubicus* (Oksman-Caldentey et al. 1987). Similarly significant variation was found for scopolamine content among somaclonal variants of *Hyoscyamus nubicus* (Oksman-Caldentey et al. 1986). Somaclonal variants for high herb, oil yield and high geraniol content in the oil over donor plants were selected in *Cymbopogon martini* (Patnaik et al. 2009). The success of somaclonal variation has been
demonstrated with the development of commercially grown cultivars such as a sugarcane variety Co94012 was developed which possessed desirable quantitative and qualitative characters over the parental genotype and the somaclonal variants were characterized by RAPD fingerprinting (Tawar et al. 2008).

The molecular mechanisms underlying somaclonal variation have been attributed to chromosome breakage, single base changes, change in copy number of repeated sequence and alteration in DNA methylation patterns, etc. (Schellenbaum et al. 2008, Kaeppler and Phillips 1993, Peredo et al. 2006). The polymorphism in the amplified products may also occur from changes in the sequence of the primer binding site (e.g. point mutations) or changes which alter the size or prevent the successful amplification of the target DNA (e.g. insertion, deletion, inversions) as suggested by Rani et al. (1995). Higher AFLP variation was found among somaclonal variants of grapevine (Schellenbaum et al. 2008). Saker et al. 2000 employed isozyme analysis and RAPD fingerprint as descriptive markers for date palm biodiversity in tissue culture derived plants. The use of PCR-based RAPD technique has been applied successfully to detect somaclonal variation in several plant species such as Lolium (Wang et al. 1993), Allium sativum L (Al-Zahim et al. 1999) as well as woody dicotyledonous species, such as Picea abies (Heinze and Schmidt 1995) and vegetable crop plants such as tomato (Soniyaya et al. 2001) and potato (Bannaceur et al. 1991). Somaclonal variation has been reported and successfully used for selection of variants with high levels of tolerance/ resistance to abiotic and biotic stresses in crop plants (Chauhan and Singh 1995). The occurrence of somaclonal variants for Picroside-1 content in P. kurroa coupled with DNA fingerprint polymorphisms confirm that regeneration of plantlets from callus cultures induces genetic changes in the genomes of cells and these genetic changes can be potentially useful, if selected for desirable traits. The vegetative propagation of P. kurroa provides added advantage of utilizing high Picroside-1 content variants because some changes in tissue cultures have been reported to revert back during sexual reproduction.