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## RESEARCH ARTICLE

# NOVEL STUDY ON *IN VITRO* CULTURE OF *RHEUM SPICIFORME* ROYLE*:* AN ENDANGERED MEDICINAL PLANT OF GUREZ VALLEY

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**ABSTRACT**

*Rheum spiciforme (polygonaceae)* is one of most known medicinal plant and is found on high altitudes of Gurez valley (3000m to 5000m). It is of high demand due to its medicinal value and has become endangered due over exploitation. *Rheum spiciforme* being a medicinal plant of immense importance with large pharmacological applications and it has been used as an ingredient of many herbal formulations, which are used for the cure of various ailments, in particular the regulation of blood pressure, fatty liver, hepatitis, fever and cancer. Tissue culture protocol was developed as a mode of the conservation. The phytohormones used for micropropagation were 6-benzyl amino purine, Kinetin, Indole - 3 acetic acid, Napthalene Acetic Acid , Indole 3-butyric acid, 2,4 – dichlorophenoxyacetic acid, Zeatin, Thidiazuron (BAP, Kn, IAA,NAA,IBA,2,4-D, Z,IAA and TDZ) which showed good results. Different parts (seeds, leaves, shoots and rhizomes) of *Rheum spiciforme* were used for *invitro* purposes. The complete germination and plantlet formation was obtained from seeds as explants (shooting/rooting) when cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combination of auxins and cytokinins.



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## INTRODUCTION

*Rheum* is one of the famous medicinal plant and belongs to the family *Polygonaceae* of group Monochlamydae of Dicots, is world over represented by 60 species (Anjen *et al.,* 2003), of which only 7 species have been reported from the Indian subcontinent (Ganie *et al.,* 2014). A total of 560 plant species of India are included in Red List of Threatened species by IUCN including *Rheum* species (Phartyal *et al.*, 2002). *Rheum species* is included in critically endangered species. Thus, identified as top priority species for conservation and cultivation (Malik *et al*., 2011a). Due to endangered nature, the export of *Rheum species* are banned or need special export permit (Jain and Sastry 1980). *R. spiciforme* is also used in the treatment of boils, wounds, rheumatic pain. Recent research has also proved that *Rheum spiciforme* has anti cancer and anti oxidative properties. Roots are frequently used for the treatment of bone fractures, backache and joint pain (Ganie *et al.,* 2014). It is also used as an adultrant. The usage of rhubarb has now been extended into the functional food as health foods (Miao, 2001). Due to a large number of medicinal properties, these species have excessive demand leading to indiscriminative and non systematic collection of these medicinal plants by pharmaceutical companies for their active constitutes and by ethnic people for domestic and traditional herbal mixture which has put severe pressure on the availability of these plants. In Kashmir Himalaya, it ranges from an altitude of 1700m to 5500m. *Rheum spiciforme* is a perennial plant and suitable for: medium (loamy) and heavy (clay) soils, prefers well-drained soil and can grow in heavy clay soil. It can grow in semi-shade (light woodland) or no shade prefers moist soil, flowers in June, and the seeds ripen in August. The leaves are large, rounded or heart-shaped, 15-30 cm across, thick and leathery, becoming red-brown on maturity. Leaves are carried on very stout stalks, 5-15 cm long, which also turn reddish. Flowers are greenish yellow and are borne in 1-3 erect cylindrical spikes, 5-30 cm tall. The flowers are hermaphrodite (have both male and female organs) and are pollinated by Wind. The local name of *R. spiciforme* is Pumbhaak and Jarochotal. The *Rheum spiciforme* has been first time gone under tissue culture process for micropropagtion for conservation purposes. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soils. Thus mass multiplication of disease free planting

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material is a general problem. In this regard, the micropropagation holds significant promise for true to type, rapid and mass multiplication and conservation of valuable genotype under disease free conditions (Sen and Sharma, 1991). Cultivation of plant tissue in synthetic media offers an alternative way of producing metabolites of interest to the traditional cultivation in the fields or greenhouses (Dornenburgh and Knorr 1995; Stockigt *et al.*, 1995; Bourgaud *et al.,* 2001; Ramachandra and Ravishankar 2002). In successful cases, cell suspension cultures can offer a repeatable method to produce secondary metabolites from elite mother plants with easily controlled conditions and with a continuous supply of material. The production of secondary metabolites can be enhanced using bioreactors. Genetic transformation may provide increased and efficient system for *in vitro* production of secondary metabolites. Tissue culture protocols have been developed for several medicinal plants, but there are many other species, which are over exploited in pharmaceutical industries and need conservation. For conservation, the tissue culture is best method. Tissue culture has now become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells and protoplasts under precisely controlled physical and chemical conditions. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soils. Thus mass multiplication of disease free planting material is a general problem. In this regard, the micropropagation holds significant promise for true to type, rapid and mass multiplication, and conservation of valuable genotype under disease free conditions (Sen and Sharma, 1991)

## MATERIALS AND METHODS

The explants taken from field material, including seeds of *R. spiciforme* were first washed with tap water to remove the dirt. Then transferred to the beaker, labolene and tween-20, a wetting agent, was poured and kept for 20 minutes. After 20 minutes, the beaker was covered with muslin cloth and was taken under the running tap water so that all the labolene or tween-20 is washed out from explants. Then these were continuously shaken for proper cleaning and were rinsed in pre-autoclaved distilled water. The cleaned plant material was subjected to brief 70% alcohol rinse and soaked in different sterilant solutions of $HgCl_2$ under laminar air flow cabinet for different time durations to get complete removal of biological contaminants. Seeds were also soaked in water and kept in refrigerator for 3 days. After 3 days, these were taken out, thoroughly washed with running tap water after cleaning them with detergent (labolene) and a few drops of tween-20 (surfactant). The powder form of MS was used in present study. In powder form of MS medium, sucrose and agar are absent. These are useful for propagation of plant species requiring nutrients according to the recipe of standard media. In the media, DDW and sucrose was added as per instructions e.g, 30.0 g of sucrose in 1 litre of MS media. Then pH (~5.8) was balanced by using NaoH (0.1N) and HCl (0.1N). After balancing the pH, agar was added as per instructions i.e. 8.0 g /L in MS media. All the glassware, forceps, petriplates, cotton plugs, DDW and beakers were autoclaved before using for tissue culture purposes. After boiling the media, the media was put into the glass vials (100ml) and were also autoclaved for 20 min. at 121°C and 15 lbs/ (inch) $^2$. Sterilized explants were inoculated onto aseptic nutrient medium under laminar air flow cabinet. The whole process of inoculation was performed under highly aseptic conditions.

After inoculation the cultures were kept in culture room. Cultures were kept for incubation under cool fluorescent tubes at day night regime of 16 hour photoperiod with light intensity of 1500 – 3000 lux at a constant temperature of 25 ± 3 ºC. Relative humidity between 60 – 70% was maintained. Subculturing was done after every 3-5 weeks, depending upon the organogenesis and proliferation potential of the explants of culture and was carried out in the Laminar air flow chamber under aseptic conditions. The plantlets obtained from different explants through repeated sub-cultures were finally left in culture vials with open mouth for three days in the incubation room, transferred to plastic pots containing soil-peat mixture and then taken out of incubation room of the lab. Attempts were made to acclimatize plants under the green house facility. Ten replicates were taken for each treatment and observations were recorded at the end of every week. Data were subjected to analysis of variance using SSPP software version 17.0 (SAS Institute Inc., Cary, NC). The growth response of explants under normal conditions and also the effect of plant growth hormone response (PGPR) treatments were considered significant according to the magnitude of the F value ($P \leq 0.005$).

## RESULTS

The different explants from field materials (leaf, leaf midribs, petioles, rhizomes, roots and shoot tips) were washed in labolene and tween 20 and then washed under running water for 20 minutes followed by surface sterilization through Mercuric chloride ($HgCl_2$) (Table 1). Effect of MS medium with different concentrations of cytokinins and auxins on callus formation from field grown plant was noted on different combinations but the best result among all was observed on 10µM BAP+8µM TDZ+8µM 2, 4-D; 15µM BAP+10µM IAA+7µM 2,4-D and on 15µM BAP+10µM IBA (Figure a and b). The callus obtained was further taken for sub-culturing on the same combinations of phytohormones.

### Germination of Seeds of *R. spiciforme*

Seed Germination studies were carried out using MS medium with different concentrations of phytohormones. Effect of MS medium with different concentrations of cytokinins and auxins on seed germination was noted i.e. 70% on 12µM BAP+10µM TDZ+10µM IBA as shown in Figure c. 80% seed germination along with complete seedling formation was observed on 15µM BAP+12µM TDZ+10µM 2,4-D as shown in Figure d. The best combinations of these phytohormones are shown in Table 2. The well established seedlings of *R. spiciforme* were further used for regeneration studies as source of various explants i.e. leaf, leaf midrib, petiole and shoot tip. The callus formed was brown and soft. The 80 % of leaf explants showed callus formation on 15µM BAP+10µM2,4-D+10µM IBA. The callus observed on these concentrations was green, soft and friable as shown in Figure e. The weight of callus was in the range of 5.0g to 7.0g. The 80% of leaf midrib explants showed callus formation on 12µM BAP+10µM 2,4-D.

**Table 1. The sterilization and contamination**

| S. No | Explants (Field Material) | Chemical sterilants (Conc.) % | Duration (min) | Contamination (%) | Explant survival %) |
|---|---|---|---|---|---|
| 1 | Seeds | $HgCl_2$ (0.02) | 10 | 30 | 80 |
| | | $HgCl_2$ (0.05) | 7 | 20 | 100 |
| | | $HgCl_2$ (0.05) | 5 | 40 | 90 |
| 2 | Leaves | $HgCl_2$ (0.02) | 10 | 70 | 50 |
| | | $HgCl_2$ (0.03) | 10 | 40 | 80 |
| | | $HgCl_2$ (0.03) | 5 | 30 | 100 |
| 3 | Petioles | $HgCl_2$ (0.01) | 10 | 80 | 50 |
| | | $HgCl_2$ (0.05) | 5 | 30 | 70 |
| | | $HgCl_2$ (0.05) | 5 | 20 | 100 |
| 4 | Leaf Midribs | $HgCl_2$ (0.02) | 10 | 30 | 80 |
| | | $HgCl_2$ (0.03) | 10 | 30 | 70 |
| | | $HgCl_2$ (0.05) | 7 | 30 | 100 |
| 5 | Shoot tips | $HgCl_2$ (0.1) | 8 | 30 | 80 |
| | | $HgCl_2$ (0.1) | 10 | 20 | 90 |
| | | $HgCl_2$ (0.05) | 7 | 30 | 100 |

**Table 2. Seed germination on different phytohormones**

| S.No. | Phytohormones(Conc.) µM | %seed germination | %Plant formation | %response |
|---|---|---|---|---|
| 1. | 10.0 µM 2,4-D | 40 | 30 | 50 |
| 2. | 10.0 µM2,4-D +10.0 µM NAA | 40 | 40 | 50 |
| 3. | 12.0 µM IAA+10.0 µM IBA | 30 | 20 | 40 |
| 4. | 10.0 µM BAP | 50 | 40 | 60 |
| 5. | 12.0 µM BAP +10.0 µM KN | 60 | 50 | 60 |
| 6. | 12.0 µM BAP+10.0 µM TDZ | 60 | 50 | 70 |
| 7. | 12.0 µM BAP+10.0 µM TDZ+10 µMIBA | 60 | 50 | 70 |
| 8. | 15.0 µM BAP +12.0 µM TDZ+10 µM 2,4-D | 70 | 60 | 80 |

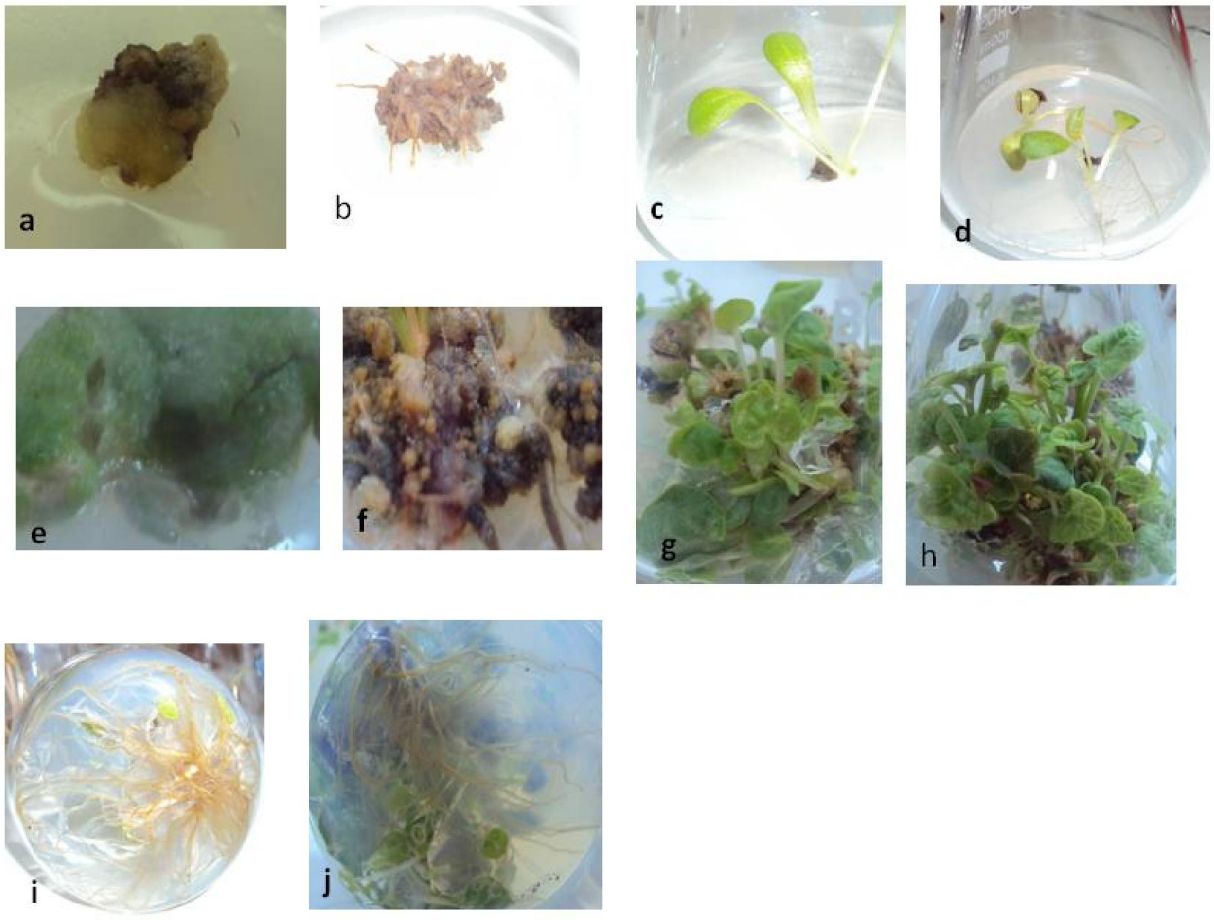


**Fig. 1. Callus formation from field material, Seed germination, callus and plant formation on MS medium with different concentrations of cytokinins in combination with auxins explants in *R. spiciforme and In-vitro* plants in nethouse for hardening**

**Table 3. Organogenesis on MS medium with different concentrations of phytohormones on multiple shoot regeneration/rooting from different explants *(in-vitro)* of *Rheum spiciforme***

| No. | Callus source | Auxins(μM) | Cytokinins(μM) | Shoot No. | Shoot length | Leaf No. | Root No. | Root length | Response | %Response |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Leaf | 12.0 (2,4-D) | - | $^{b}$4.2±0.5 | $^{b}$2.3±0.4 | $^{b}$5.3±0.3 | $^{b}$2.5±0.5 | $^{a}$1.8±0.4 | shooting/rooting | 60 |
| 2 | - | 15.0 (2,4-D) | - | $^{b}$4.8±0.5 | $^{b}$2.5±0.5 | $^{b}$5.5±0.5 | $^{b}$2.9±0.5 | $^{ba}$2.2±0.5 | do | 60 |
| 3 | - | 12.0 (NAA) | - | $^{a}$3.6±0.5 | $^{a}$1.9±0.5 | $^{a}$4.3±0.5 | $^{b}$2.6±0.3 | $^{a}$1.8±0.3 | do | 60 |
| 4 | - | 15 NAA | - | $^{b}$4.5±0.5 | $^{b}$2.4±0.3 | $^{b}$5.2±0.3 | $^{c}$3.6±0.5 | $^{b}$2.6±0.3 | do | 60 |
| | - | 12 IAA | - | nil | nil | nil | $^{d}$4.7±0.5 | $^{b}$2.8±0.3 | direct rooting only | 50 |
| | - | 15 IAA | - | nil | nil | nil | $^{e}$5.3±0.5 | $^{c}$3.5±0.5 | do | 50 |
| | - | 12 IBA | - | nil | nil | nil | $^{e}$5.6±0.4 | $^{c}$3.5±0.5 | do | 50 |
| | - | 15 IBA | - | nil | nil | nil | $^{e}$5.7±0.5 | $^{c}$3.8±0.5 | do | 50 |
| 5 | - | - | 12 BAP | $^{c}$5.6±0.3 | $^{b}$2.4±0.4 | $^{c}$6.5±0.5 | nil | nil | direct shooting only | 60 |
| 6 | - | - | 15 BAP | $^{d}$6.2±0.4 | $^{b}$2.5±0.5 | $^{d}$7.3±0.5 | nil | nil | do | 70 |
| 7 | - | - | 12 TDZ | $^{c}$5.2±0.3 | $^{b}$2.5±0.3 | $^{c}$6.2±0.5 | $^{b}$2.3±0.3 | $^{a}$1.5±0.4 | direct shooting/rooting | 70 |
| 8 | - | - | 15 TDZ | $^{c}$5.6±0.4 | $^{b}$2.4±0.2 | $^{c}$6.4±0.3 | $^{b}$2.4±0.5 | $^{a}$1.9±0.3 | do | 70 |
| | - | - | 12 KN | $^{a}$5.3±0.2 | $^{a}$1.4±0.2 | $^{a}$5.9±0.2 | $^{a}$1.9±0.2 | $^{a}$1.5±0.2 | do | 70 |
| 9 | - | - | 15 KN | $^{c}$5.5±0.3 | $^{a}$1.9±0.3 | $^{c}$6.3±0.2 | $^{b}$2.3±0.2 | $^{a}$1.7±0.2 | do | 70 |
| 10 | - | 15 2,4-D | 15 BAP | 9.2±0.5 | $^{c}$3.9±0.5 | $^{f}$10.4±0.5 | $^{c}$3.9±0.2 | $^{c}$3.3±0.2 | multiplication/elongation/rooting | 80 |
| 11 | - | 15 NAA | 15 BAP | $^{e}$8.8±0.5 | $^{c}$3.5±0.2 | $^{e}$9.5±0.3 | $^{d}$4.5±0.3 | $^{c}$3.4±0.5 | do | 80 |
| 12 | - | 15 IBA | 15 BAP | $^{f}$9.6±0.5 | $^{c}$3.9±0.3 | $^{f}$10.9±0.5 | $^{e}$6.3±0.5 | $^{d}$4.5±0.3 | do | 80 |
| 13 | - | 15 IAA | 15 BAP | $^{f}$9.1±0.5 | $^{c}$3.2±0.3 | $^{f}$10.4±0.5 | $^{e}$6.1±0.5 | $^{d}$4.1±0.3 | do | 80 |
| 13 | Mid rib | 12.0 (2,4-D) | - | $^{b}$4.5±0.3 | $^{b}$2.5±0.4 | $^{b}$5.5±0.5 | $^{b}$2.6±0.5 | $^{b}$2.2±0.5 | direct shooting/rooting | 60 |
| 14 | - | 15.0 (2,4-D) | - | $^{c}$5.5±0.4 | $^{b}$2.9±0.5 | $^{c}$6.2±0.5 | $^{c}$3.2±0.5 | $^{b}$2.5±0.4 | do | 70 |
| 15 | - | 12.0 (NAA) | - | $^{a}$3.7±0.4 | $^{b}$2.3±0.5 | $^{a}$4.6±0.5 | $^{b}$2.4±0.5 | $^{b}$2.3±0.2 | minimum shooting/rooting | 60 |
| 16 | - | 15 NAA | - | $^{b}$4.7±0.5 | $^{b}$2.6±0.4 | $^{b}$5.5±0.4 | $^{c}$3.8±0.5 | $^{b}$2.9±0.5 | do | 60 |
| | - | 12 IAA | - | nil | nil | nil | $^{e}$5.2±0.5 | $^{b}$2.7±0.5 | direct rooting only | 50 |
| | - | 15 IAA | - | nil | nil | nil | $^{e}$5.4±0.5 | $^{c}$3.3±0.5 | do | 50 |
| | - | 12 IBA | - | nil | nil | nil | $^{e}$5.3±0.7 | $^{c}$3.5±0.5 | do | 50 |
| | - | 15 IBA | - | nil | nil | nil | $^{e}$5.7±0.5 | $^{c}$ 3.8±0.5 | do | 50 |
| 17 | - | - | 12 BAP | $^{c}$5.9±0.5 | $^{b}$2.6±0.4 | $^{c}$6.8±0.5 | nil | nil | direct shooting only | 70 |
| 18 | - | - | 15 BAP | $^{d}$6.6±0.5 | $^{b}$2.8±0.5 | $^{d}$7.7±0.5 | $^{a}$1.9±0.4 | $^{b}$2.3±0.2 | diirect shooting/rooting | 70 |
| 19 | - | - | 12 TDZ | $^{c}$5.5±0.3 | $^{b}$2.6±0.3 | $^{c}$6.6±0.5 | $^{b}$2.9±0.4 | $^{a}$1.8±0.5 | do | 70 |
| 20 | - | - | 15 TDZ | $^{c}$5.9±0.5 | $^{b}$2.8±0.5 | $^{c}$6.9±0.3 | $^{b}$2.9±0.5 | $^{b}$2.2±0.3 | do | 70 |
| | - | - | 12 KN | $^{c}$5.5±0.3 | $^{a}$1.5±0.1 | $^{c}$6.2±0.2 | $^{a}$1.7±0.3 | $^{a}$1.8±0.1 | do | 70 |
| 21 | - | - | 15 KN | $^{c}$5.8±0.3 | $^{b}$2.2±0.1 | $^{c}$6.5±0.2 | $^{b}$2.6±0.4 | $^{b}$2.3±0.2 | do | 70 |
| 22 | - | 15 2,4-D | 15 BAP | $^{f}$9.5±0.5 | $^{dc}$4.1±0.1 | $^{f}$10.6±0.5 | $^{d}$4.3±0.2 | $^{c}$3.5±0.5 | multiplication/elongation/rooting | 80 |
| 23 | - | 15 NAA | 15 BAP | $^{f}$9.3±0.2 | $^{c}$3.7±0.5 | $^{e}$9.9±0.4 | $^{d}$4.5±0.2 | $^{c}$3.7±0.4 | do | 80 |
| 24 | - | 15 IBA | 15 BAP | $^{g}$10.3±0.4 | $^{d}$4.3±0.2 | $^{g}$11.2±0.5 | $^{e}$6.5±0.4 | $^{d}$4.7±0.5 | do | 80 |
| 25 | - | 15 IAA | 15 BAP | $^{f}$9.8±0.4 | $^{d}$4.1±0.2 | $^{f}$10.8±0.5 | $^{e}$6.1±0.4 | $^{d}$4.3±0.5 | do | 80 |
| 25 | Petiole | 12.0 (2,4-D) | - | $^{a}$3.8±0.4 | $^{a}$1.8±0.4 | $^{a}$4.9±0.5 | $^{b}$2.5±0.3 | $^{a}$1.7±0.4 | minimum shooting/rooting | 60 |
| 26 | - | 15.0 (2,4-D) | - | $^{b}$4.7±0.5 | $^{b}$2.3±0.2 | $^{b}$5.5±0.6 | $^{b}$2.6±0.3 | $^{a}$1.9±0.4 | do | 60 |
| 27 | - | 12.0 (NAA) | - | $^{a}$3.5±0.4 | $^{a}$1.7±0.3 | $^{a}$4.3±0.2 | $^{b}$2.4±0.3 | $^{a}$1.7±0.5 | do | 60 |
| 28 | - | 15 NAA | - | $^{b}$4.2±0.5 | $^{b}$2.3±0.4 | $^{a}$4.9±0.4 | $^{c}$3.5±0.4 | $^{b}$2.6±0.3 | do | 60 |
| | - | 12 IAA | - | nil | nil | nil | $^{d}$4.3±0.5 | $^{b}$2.6±0.4 | direct rooting only | 50 |
| | - | 15 IAA | - | nil | nil | nil | $^{d}$4.9±0.6 | $^{c}$3.1±0.5 | do | 50 |
| | - | 12 IBA | - | nil | nil | nil | $^{e}$5.2±0.3 | $^{c}$3.3±0.5 | do | 50 |
| | - | 15 IBA | - | nil | nil | nil | $^{e}$5.5±0.5 | $^{c}$3.6±0.5 | maximum direct rooting only | 50 |
| 29 | - | - | 12 BAP | $^{c}$5.6±0.5 | $^{b}$2.5±0.4 | $^{c}$6.4±0.5 | nil | nil | | |
| 30 | - | - | 15 BAP | $^{c}$5.9±0.6 | $^{a}$1.6±0.5 | $^{d}$7.4±0.5 | nil | nil | direct shooting only | 70 |
| 31 | - | - | 12 TDZ | $^{c}$5.2±0.1 | $^{b}$2.2±0.1 | $^{c}$6.3±0.5 | $^{a}$1.8±0.3 | $^{a}$1.5±0.3 | direct shooting/rooting | 70 |
| 32 | - | - | 15 TDZ | $^{c}$5.5±0.3 | $^{b}$2.4±0.4 | $^{c}$6.4±0.5 | $^{b}$2.4±0.3 | $^{a}$1.7±0.3 | do | 70 |
| | - | - | 12 KN | $^{c}$5.1±0.3 | $^{a}$1.5±0.2 | $^{b}$5.6±0.4 | $^{a}$1.8±0.3 | $^{a}$1.4±0.2 | do | 70 |
| 33 | - | - | 15 KN | $^{c}$5.4±0.3 | $^{a}$1.7±0.3 | $^{b}$5.9±0.4 | $^{b}$2.3±0.3 | $^{a}$1.7±0.3 | do | 70 |

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|---|---|---|---|---|---|---|---|---|---|---|
| 34 | - | 15 2,4-D | 15 BAP | $^{f}$9.3±0.5 | $^{c}$3.6±0.4 | $^{f}$10.4±0.5 | $^{c}$3.7±0.2 | $^{b}$2.9±0.3 | multiplication/elongation/rooting | 80 |
| 35 | - | 15 NAA | 15 BAP | $^{e}$8.7±0.5 | $^{c}$3.5±0.2 | $^{f}$10.4±0.2 | $^{c}$3.9±0.2 | $^{c}$3.3±0.2 | do | 80 |
| 36 | - | 15 IBA | 15 BAP | $^{f}$9.5±0.4 | $^{c}$3.9±0.3 | $^{f}$10.7±0.5 | $^{e}$6.3±0.2 | $^{d}$4.4±0.3 | do | 80 |
| 37 | - | 15 IAA | 15 BAP | $^{e}$8.9±0.4 | $^{c}$3.4±0.3 | $^{e}$9.9±0.5 | $^{e}$6.1±0.2 | $^{d}$4.2±0.3 | do | 80 |
| 37 | Shoot tip | 12.0 (2,4-D) | - | $^{b}$4.6±0.5 | $^{b}$2.7±0.3 | $^{b}$5.8±0.5 | $^{b}$2.8±0.3 | $^{b}$2.5±0.3 | direct shooting/rooting | 60 |
| 38 | - | 15.0 (2,4-D) | - | $^{c}$5.5±0.4 | $^{b}$2.8±0.5 | $^{c}$6.4±0.5 | $^{c}$3.4±0.2 | $^{b}$2.5±0.3 | do | 60 |
| 39 | - | 12.0 (NAA) | - | $^{b}$4.2±0.2 | $^{b}$2.3±0.5 | $^{b}$5.3±0.4 | $^{b}$2.9±0.3 | $^{b}$2.5±0.3 | minimum shooting/rooting | 60 |
| 40 | - | 15 NAA | - | $^{b}$4.8±0.5 | $^{b}$2.7±0.3 | $^{b}$5.5±0.5 | $^{d}$4.4±0.3 | $^{b}$2.9±0.5 | do | 60 |
| | - | 12 IAA | - | nil | nil | nil | 5.6±0.5 | $^{b}$2.8±0.4 | direct rooting only | 50 |
| | - | 15 IAA | - | nil | nil | nil | 5.9±0.5 | $^{c}$3.6±0.5 | do | 50 |
| | - | 12 IBA | - | nil | nil | nil | 5.8±0.3 | $^{c}$3.5±0.5 | do | 50 |
| | - | 15 IBA | - | nil | nil | nil | 6.5±0.5 | $^{c}$3.8±0.5 | maximum direct rooting | 50 |
| 41 | - | - | 12 BAP | $^{c}$5.8±0.3 | $^{b}$2.9±0.4 | $^{c}$6.9±0.5 | nil | nil | direct shooting only | 70 |
| 42 | - | - | 15 BAP | $^{d}$6.6±0.4 | $^{b}$2.9±0.4 | $^{d}$7.8±0.5 | $^{b}$2.5±0±1 | $^{a}$1.9±0.3 | do | 70 |
| 43 | - | - | 12 TDZ | $^{c}$5.6±0.3 | $^{b}$2.7±0.2 | $^{c}$6.7±0.5 | $^{a}$2.4±0.5 | $^{a}$1.8±0.2 | do | 70 |
| 44 | - | - | 15 TDZ | $^{c}$5.9±0.5 | $^{b}$2.7±0.2 | $^{c}$6.9±0.4 | $^{b}$2.7±0.5 | $^{b}$2.4±0.3 | do | 70 |
| | - | - | 12 KN | $^{c}$5.6±0.3 | $^{a}$1.7±0.2 | $^{c}$6.2±0.4 | $^{a}$1.8±0.3 | $^{a}$1.8±0.5 | do | 70 |
| 45 | - | - | 15 KN | $^{c}$5.8±0.3 | $^{b}$2.3±0.4 | $^{c}$6.6±0.5 | $^{b}$2.6±0.3 | $^{b}$2.5±0.4 | do | 70 |
| 46 | - | 15 2,4-D | 15 BAP | $^{g}$11.7±0.5 | $^{d}$4.2±0.1 | $^{h}$12.8±0.5 | $^{d}$4.3±0.2 | $^{c}$3.5±0.3 | multiplication/elongation/rooting | 80 |
| 47 | - | 15 NAA | 15 BAP | $^{e}$9.2±0.5 | $^{c}$3.8±0.5 | $^{f}$10.8±0.5 | $^{d}$4.5±0.6 | $^{c}$3.5±0.4 | do | 80 |
| 48 | - | 15 IBA | 15 BAP | $^{f}$10.3±0.2 | $^{d}$4.4±0.3 | $^{g}$11.5±0.5 | $^{e}$6.6±0.7 | $^{d}$4.7±0.5 | do | 80 |
| 48 | - | 15 IAA | 15 BAP | $^{e}$9.9±0.2 | $^{d}$4.1±0.3 | $^{f}$10.6±0.5 | $^{e}$6.3±0.7 | $^{d}$4.4±0.5 | do | 80 |

Values are represented as mean±SD (n=10), Data was analyzed by ANOVA using Duncan's multiple range test (SPSS17.0); the values with different superscript along the columns are statically significant at P<0.005 , Data scored after 12 weeks of culture period

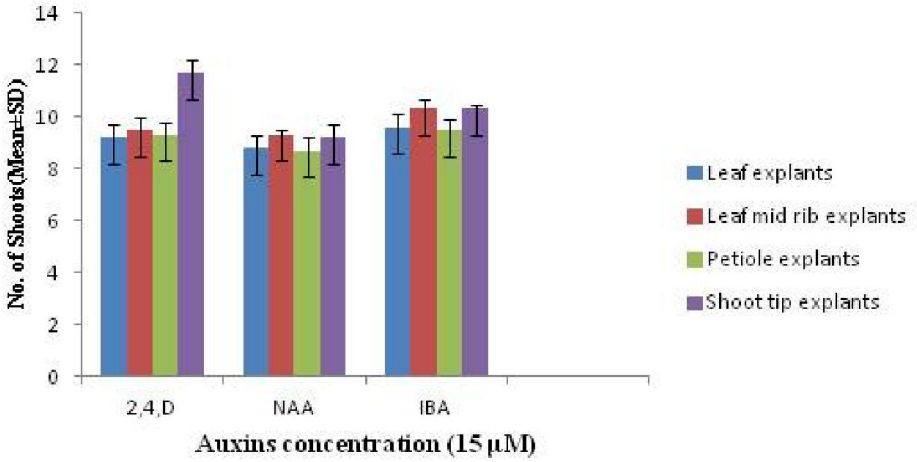


**Fig. 2. Effect of 15µM BAP with different auxins (15µM) on shoot regeneration**




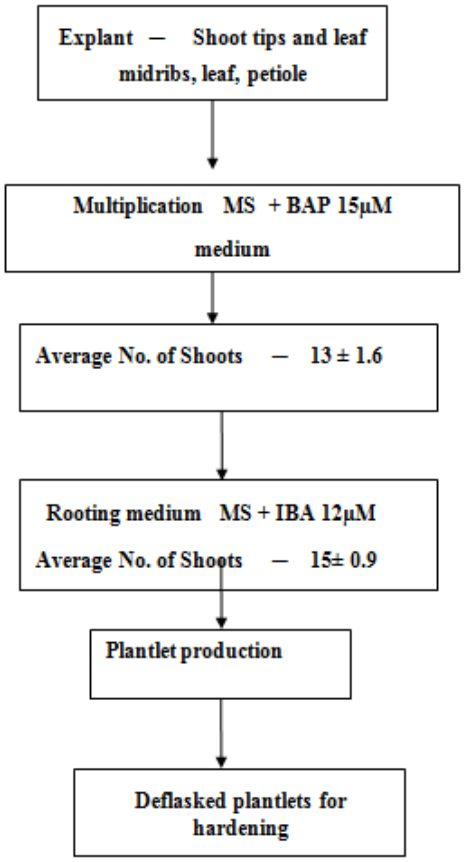


**Fig. 3. *Invitro* protocol for *R.spiciforme***

Callus was brownish and weight was 4.0 g to 6.0 g .The 80% of petiole explants showed response on callus formation on 12 µM BAP+10 µM 2,4-D and on 12 µM BAP + 10 µM NAA, which was green and soft and the weight observed was in the range of 5.0 g to 8.0 g**.** Callus formation from 80% shoot tip explants was observed on 15µM BAP+10µM 2,4-D+10 µM IBA. The callus obtained were healthy, green, friable and soft and weight observed ranges from 5.0 g to 7.0g as shown in Figure f**.** The callus formed was further used for regeneration. Different combinations of MS media were used for regeneration.

**Organogenesis from different in-vitro raised explants of R. spiciforme**

To obtain the complete plant formation and to reduce the callus formation for hardening purpose, the *in-vitro* explants were further sub-cultured on different combinations of phytohormones. The different explants used for direct regeneration were leaves, leaf midribs, petioles and shoot tips. The ***in-vitro*** explants was sub-cultured on different concentrations of phytohormones starting from 5 µM to 15 µM but the best results were found on high concentrations of phytohormones and the regeneration data collected through leaf, leaf midrib, petiole, shoot tip explants was analysed by ANOVA using Duncan's multiple test as shown in Table 3 The plant formation on auxins from explants i.e., leaf , leaf mid rib, petiole and callus was found on MS+15 µM 2,4-D and from cytokinins was found on MS+15 BAP with 80% response .On the combination of phytohormones 10 µM IBA+15 µM BAP (figure g) complete plant formation was also observed with 80% response in explants i.e, leaf. Leaf midrib and petiole where as shoot tip explants showed complete plant formation on 10 µM 2,4-D +15 µM BAP with 80% response (Figure h).

It was observed that only rooting was formed on IAA and IBA when used individually and the rooting in large number was found on 15 µM IBA with 70% response (Figure i) in all the explants. It was also observed that all the four explants showed no rooting on low concentrations of BAP and KN (Figure j). The average number of regenerated shoots was 9 to 10 shoots per explant. However, maximum number of regenerated shoots 12 shoots per explants was observed in 15µM BAP + 15µM 2, 4-D from shoot tip explants (Figure 2)

**Hardening and Acclimatization**

The tissue cultured raised plants were used for hardening. The first step for hardening was to enclose the culture vials containing *in vitro* plantlets of *Rheum spiciforme*. After enclosure of culture vials they were kept in the incubation room for two weeks to reduce the high humidity conditions within the culture vials. After two weeks these plantlets were transferred from incubation room to normal room conditions where *in-vitro* plantlets were deflasked and agar was washed carefully with DDW with the help of very soft brushing. The 20 plantlets from each of the three *Rheum spiciforme* were then potted in plastic pots containing autoclaved sand: soil: peat, in ratio of 1:2:1 mixture i.e. 4Kg sand, 8Kg soil, 4Kg peat with MS medium powder (4.2gm) per plastic pot and then these plants were covered with small transparent polythene bags. The soil and sand was taken from the net house. The potted plantlets were placed at room temperature 20-25°C for 15 days and were under manual misting on alternate days as shown in Figure k. After 15 days the polythene bags were removed from the plastic pots and then these plants were shifted to net house. In net house these were watered properly for 10 days as shown in Figure l. After 10 days the plants were taken out from the pots and were sown directly in the soil of beds of nursery. In beds, the *in-vitro* plants also showed good growth response 35% in *R. spiciforme* as shown in figure m. The flow chart shows the whole protocol of *R.spicoforme* (Figure 3).

## DISCUSSION

In present studies, the main aim was the micro-propagation of the three species of *Rheum spiciforme* as they are vulnerable and now endangered species. They have vanished from lower altitudes and now found only on high altitudes of Kashmir Himalaya, so thae micropropagation is best step towards conservation of *Rheum*. In this study the micropropagation of *R. spiciforme* has been done for the first time. *Rheum spiciforme* showed good response i.e. 80 % to 90% response. Seeds were soaked in DDW for three days and kept in refrigerator followed by sterilization with $HgCl_2$. The best result in this study was found on 0.05% $HgCl_2$ for a period of 15 minutes. But, according to Farzami *et al*. (2005) sterilization of seeds of *R. ribes* L were achieved after immersing them in 0.3% of benomil solution for 1 h, and then transferred to ethanol for 1minute and then to NaOCl (50%) for10 minutes. The shoot tips showed 80% response on 0.05% $HgCl_2$ when kept for 10 minutes in present studies, but in earlier reports of Lal and Ahuja(1989), the sterilization of shoot tips of *R. emodi* were achieved with 70% alcohol for 30 seconds followed by 0.1% $HgCl_2$ for a period of 45-60 seconds and 100% sterilization of leaves was obtained at 0.1% $HgCl_2$ treated for 7 minutes. However, in our studies the leaves sterilization was found to be good using 0.02% $HgCl_2$ for 5 minutes. it was observed that the seeds of *R. spiciforme* showed 100% germination on MS medium when treated with different phytohormones but according to Farzami *et al*.(2002 and 2005) who also reported the seedling formation on MS basal medium in *R. ribes* L. which was chilled for three days with GA3 also. But this protocol was opposite to our protocol as no GA3 was used. So, in present study our results were good as the seeds showed good response directly without using GA3. In present studies, *R.spiciforme* showed good response on different concentration as this species was first time used in tissue culture techniques. The shoot regeneration was observed on 15 µM 2,4-D, 15 µM BAP, 15 µM TDZ and also on 15 µM Kinetin. But the maximum shoot regeneration was formed on 15 µM BAP and on combination of 15 µM BAP with 15 µM 2, 4-D. Shoot multiplication on MS medium from shoot tips and leaf was also observed when the medium was supplemented with different concentrations of BAP, Kinetin or TDZ .Similar results were obtained given by Walkey and Mathews (1979), Roggemans and Boxus (1988) on shoot formation in *R. rhaponticum* on MS medium when supplemented with Kinetin; but Lal and Ahuja (1989) reported that Kinetin did not show any response when shoot tips of *R. emodi* were cultured on it. This shows that the results in present studies was good as shoot regeneration was observed on Kinetin.

Elongation of shoots was also observed in all these three species with different phytohormones. Number of shoots, size of shoots and leaf showed increase with increase in concentration of phytohormones. The maximum length in *Rheum* species *was* observed on BAP 15 µM when combined with IAA 15 µM, at BAP 15 µM when combined with IBA 15 µM and at Kinetin, TDZ, 2,4-D and NAA also on 15 µM combined with IAA and IBA showed maximum shoot formation and elongation in *Rheum spiciforme.* These results are similar to the reports of Hu Wang (1983) and with Wareing and Philips (1981), according to them the inhibitory effect of cytokinin is expected. Presently, it was also observed that that the increase in concentration of phytohormones the shoot regeneration was also increased but in case of IAA an IBA no shoot regeneration was formed even on high concentration in all *R.spiciforme*. It was observed that without changing the medium the cultures of *R. spiciforme* were conserved for more than one year on MS medium supplemented with different phytohormones especially on BAP and IBA and 2,4-D. This conservation technique of medium term enabled the extension of subculture period upto one year which is in agreement with Ashmore (1997). Same report was given by Holobiuc *et al*. (2004, 2006) reported the medium term conservation of some of the endangered species of *Artemesia tsechernieviana* and *Astragalus pseudopurpureus*.

Rooting on MS with different phytohormones was also observed in present studies. But rooting on basal medium without using any phytohormones was according to the reports of Walkey and Mathews (1979), who initiated the roots in *R. rhaponticum* using MS basal medium; Malik *et al*. (2009) who reported similar observations in *R. emodi,* where as in present studies, the maximum rooting was observed on IBA and IAA. Besides rooting was also observed on 2,4-D and NAA. These

results are similar to that of Rogemans and Claes (1979) and Roggmans and Boxus (1988) who initiated roots in *R. rhaponticum* on MS + IBA, Lal and Ahuja (1989 and 1993) in *R. emodi* on MS + IBA; Kanji *et al.* (1990) in *R. palmatum* on MS +NAA; Thomas *et al.* (2005) on MS +IAA in *R. rhubarbarum, R. rhaponticum, R. undulatum* but the reports of Farzami *et al*. (2005) showed the formation of maximum roots on half strength MS+IBA in *R. ribes*. When BAP was used on high concentration i.e. 15 µM, shooting as well as minimum rooting was formed in all these three species was observed in present studies. Similarly when 2, 4-D and NAA was used in high concentration minimum shooting and maximum rooting was observed. It was noticed in present studies that IAA and IBA showed good response for rooting and BAP showed good response for shoot formation in all the three species and this result is similar to the report given by Parveen *et al*. (2012) to some extent. However , no callus or shoot formation was observed from roots or rhizomes of all the three *Rheum spiciforme* but Rashid *et al.* (2014) reported callus formation from the rhizome explants were inoculated on MS medium containing different concentrations of auxins either individually or in combination, 2, 4-D. The *in-vitro* raised plantlets were transferred to Plastic pots containing potting mixture of sterilized sand: soil: peat: (1:2:1) with MS medium (4.2 gm) and were covered with transparent polythene bags and was placed in culture room for 15 days. These results are same with that of Lal and Ahuja (1989 and 1993) who also reported hardening of *in-vitro* raised plantlets of *R. emodi* with 89%-90% survival rate, Walkey and Mathews (1979) who also reported establishment of rooted plantlets of *R. rhaponticum* in peat pots, and also with the findings of Roggemans and Boxus (1988) who reported about 90% survival rate in *R .rhaponticum*.

**Conclusion**

This study is first report of micropropgation at world level for *R.spiciforme* and was collected from Gurez valley. The first reproducible protocol of tissue culture was developed for the conservation purposes vis a vis good survival rate in green house.

**Conflict of Interest**

The authors hereby declare that they have no conflict of interest.

**Author's contributions**

All authors equally participated in designing experiments analysis and interpretation of data. All authors read and approved the final manuscript.

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