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Original Article

Effect of Plant Growth Regulator on *In vitro* Multiplication of Turmeric (*Curcuma longa* L. cv.Ranga)

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Abstract

High frequency *in vitro* plantlet regeneration method was developed for *Curcuma longa* L. (cv.Ranga) using fresh sprouting rhizome bud on semisolid culture media. The explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentration and combinations of BAP (6-Benzyl-amino-purine) and NAA(α- Naphthalene acetic acid) for shoot and root induction. Explants cultured on MS basal medium supplemented with 2.0mg/l BAP+0.5gm/l NAA showed highest rate of shoot multiplication. *In vitro* shoots were rooted on to the half-strength MS basal media supplemented with 2.0 mg/l NAA and rooting was better. Rooted shoots were transplanted in the green house for hardening and their survival rate was 95% in the field condition.
Keywords: *Curcuma longa* L. (cv.Ranga), Micropropagation, Plant growth regulator, Sprouting rhizome bud, Tissue culture

Introduction

Turmeric (*Curcuma longa* L. cv.Ranga) a perennial herb and an important spice, belong to family *Zingiberaceae*. The plant grown to a height of three to five feet, and is cultivated extensively in Asia, India, China, and other countries with a tropical climate. It has oblong, pointed leaves and bears funnel-shaped yellow flowers (Hand book of Horticulture, 2003). The rhizome is the portion of the plant used medicinally; it is usually boiled, cleaned, and dried, yielding a yellow powder. Dried rhizome of *Curcuma longa* is the source of the spice turmeric, the ingredient that gives curry powder its characteristic yellow color. Turmeric is used extensively in foods for both its flavor and color. Turmeric has a long tradition of use in the Chinese and Ayurvedic systems of medicine, particularly as an anti-inflammatory agent, and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic. Turmeric can also be applied topically in poultices to relieve pain and inflammation. Current research has focused on turmeric's antioxidant, hepato-protective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders (Ammon and Wahl, 1991, Kiso *et al*.,1983).

The active constituents of turmeric are the flavonoid, curcumin and volatile oils including tumerone, atlantone, and zingiberone. Other constituents include sugars, proteins, and resins (Roses,1999). The best-researched active constituent is curcumin, which comprises 0.3 to 5.4 percent of raw turmeric. Pharmacokinetic studies in animals demonstrate that 40-85 percent of an oral dose of curcumin passes through the gastrointestinal tract unchanged, with most of the absorbed flavonoid being metabolized in the intestinal mucosa and liver. Due to its low rate of absorption, curcumin is often formulated with bromelain for increased absorption and enhanced anti-inflammatory effect. Curcumin, a major bio-reactive secondary metabolite obtained from the rhizome of turmeric, which is anticarcinogenic, is now being used in anticancer drug development programmes (Sakamura *et al*., 1986) Leaves and stems of turmeric are also used as biofertilizer. In India, ethnologically, turmeric occupies an important position in our life, forming an integral part of rituals, ceremonies and cuisine (Holttum,1950). In Orissa the turmeric cultivated variety Ranga of Kandhamala enjoys a monopoly in its production and export to different parts of the country. In conventional method turmeric is propagated vegetatively by perennial rhizomes. Low productivity, disease susceptibility and higher cost of seed rhizomes production are major constraints faced by the farmers. Turmeric rhizomes have a dormancy period and only sprout during monsoon. A large amount of the edible part (rhizome) is stored for stock purpose for the next season. Maintenance of germplasm by annual planting is expensive and labor intensive for the marginal farmers.

Moreover, diseases such as rhizome rot, caused by *Pythium* species and leaf spot, caused by species of *Taphrina* and *Collectrichum,* take a heavy toll during storage and in the field, there by causing a severe shortage of healthy planting materials.

In the recent decade's micro-propagation techniques are being profitably used to over come such constraints in various vegetatively propagated crop as well as ornamental and horticultural plants. Considering high demand and greater economic and medicinal value of turmeric, it is necessary to develop a suitable farmer friendly protocol for mass production of disease free stocks through tissue culture technique. There are many reports on *invitro* propagation of some rhizomatous plants like Ginger, Cardamom and *Alpania calcarata* (Yasuda et al.1987, 1988). The tissue culture protocols for turmeric have also been reported by few workers . How ever in Orissa no previous work was done on *invitro* propagation of Ranga cultivated variety of Turmeric. In the present study effort was made to establish a low cost *invitro* protocol for the Ranga cultivated variety of turmeric from sprouting rhizome bud. This cultivated variety of turmeric in Orissa has a special reputation both national and international market as a best condiment. This work is obviously a first step in the advancement of turmeric tissue culture in Orissa. It is therefore highly desirable to standardize a methodology for efficient *invitro* culture to provide a year round supply of disease-free quality planting materials for large scale commercial cultivation of the crop species to meet the market demand and socioeconomic development of the marginal farmers of the state.

Materials and Methods

Explant source

Healthy rhizome sprouts with active buds were collected from the rhizome of *Curcuma longa* (cv-Ranga) maintained in the nursery bed of experimental garden of P.G. department of Botany Utkal University. They were cut in to 1.5 to 2 cm length with active buds intact. These rhizome sprouting with active buds were washed with 5% (v/v) detergent solution Teepol (Qualigen, Mumbai, India) for 10 minute and rinsed several times with running tap water. These rhizome sprouting bud cuttings were surface sterilized with bavistin 0.3% followed by streptomycin 0.2% for 10 minute and then washed with sterile distilled water and transferred to laminar air flow cabinet .In the laminar chamber the sprouting bud cuttings were again dip with 70% alcohol for 30 second to one minutes followed by another treatment in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. Finally, the sprouting bud cuttings were washed thoroughly 3 to 4 times with sterile distilled water and soaked with sterile blotting paper and used as explants for *In vitro* cultures before the inoculation in to sterilized nutrient agar media pre-packed in culture tubes (Smith and Hamill,1996).

Culture medium and condition: The sterilized blotted explants were implanted on to the Murashige and Skoog's (1962) agar-gelled medium fortified with various concentrations/combinations of growth hormones. For shoot induction, the medium was supplemented with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l BAP and 0.25 to 0.5 mg/l α-Naphthalene acetic acid (NAA) either individually or in combination. For root induction *invitro* raised shoots measuring about 4-5cm grown in multiplication medium were cultured on half-strength MS medium supplemented with either NAA (α-Naphthalene acetic acid) or IBA (Indole3-butyricacid) in concentration of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l. The pH of the medium was adjusted to 5.8 before autoclaving at 1.04 kg/cm^{-2} pressure and 121°C temperature for 15 - 20 minute. Molten medium of 20 ml was dispensed into the culture tube and plugged with nonabsorbent cotton wrapped in one layer of cheesecloth. All cultures were incubated in 16 h light/8 h dark photoperiod (cool, white fluorescent light - 30µmol $m^{-2}S^{-1}$) . The cultures were incubated at 25 ± 3°C in diffused light under 60 - 70% relative humidity in the culture room. Each treatment had 20 culture tubes and the experiment was repeated thrice. The cultures were maintained by regular subcultures at 2 weeks intervals on fresh medium with the same compositions.

Acclimatization

Rooted micro-propagules were removed from the culture tube and the roots were washed under running tape water to remove agar .Then the plantlets were transferred to sterile poly pots (small plastic cups) containing pre-soaked vermiculite (TAMIN ,India) and maintained inside growth chamber

set at temperature 28^0C and 70-80% relative humidity. After three weeks they were transplanted to earthen pots containing mixture of soil + sand + manure (FYM) in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization.

Observation of cultures and presentation of results: Twenty cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed by the Post- Hoc Multiple Comparison test at the $P < 0.05$ level of significance (Marascuilo and McSweency, 1977).

Results

The response of *Curcuma longa* (cv.Ranga) rhizome sprouting bud explants cultured on different shoot proliferation media over a period of six weeks is presented in Table.1, culture medium devoid of growth regulators (Control, Table.1) failed to stimulate the bud break response in the explants even when the cultures were maintained beyond the normal observation period of four weeks. MS medium with growth regulator (BAP and NAA) supplements produced better results in terms of percentage explants response (Fig.2. A), shoots /explant, average shoot length and average number of roots and length of roots. In such media combinations bud break was noticed within 8-10 days of culture (Table.1, Fig.1. A&B). Of the combination tested MS + BAP (2.0 mg/l) + NAA (0.5mg/l) elicited optimal response in which an average of 7.0 ± 0.18, shootlets (Fig.1.C&D,Table-1) with a mean shoot length of 5.4 ± 0.09 cm per explant was recoded followed by the second best shoot multiplication 4.5 ± 0.12 in the medium MS + BAP (1.5 mg/l) + NAA (0.5mg/l) with a mean shoot length of 4.6 ± 0.16 cm . Higher concentration of BAP (3.0mg/l) with NAA (0.5 mg/l) showed callusing of explants with fewer number of shoots .In such cultures shoots were stunted with a mean shoot length of 2.4 ± 0.24 cm. The well developed elongated shoots measuring about 4-5cm in length were excised from shoot clump and transferred to half strength MS medium containing NAA or IBA. The rooting responses of shoots on different media, which included rooting percentage, days required for root initiation mean number of roots/shoot and mean root growth over a period of three weeks were different (Table-2.Fig.2.B). There was very stunted rooting in case of shoot planted on auxin free basal medium (control). Similarly, at lower level of NAA (0.25 mg/l) treatments there was hardly any rooting in the cultured shoots during the 4 weeks of observation period. However higher concentration of NAA (1.5&2.0 mg/l) and IBA at all concentration (i.e. from 0.25.0.05, 1.0, 1.5,2.0, 2.5 and 3.0 mg/l) tested responds well. Rooting was better in the culture which had combination of $^1/_2$ MS+2.0 mg/l NAA where about 95 % cultures responded with an average number of 7.3 ± 0.32 roots per plantlet and an average root length 4.5 ± 0.12 cm was recorded (Fig.1.C&D, Table-2) .The second highest response (80%) was recorded at 1.5 mg/l of NAA (Pillai and Kumar, 1982, Sugaya 1991, Zakaria and Ibrahim, 1986). It was observed that root primordial emerged from the shoot base starting from day 8 to 10 days after shoot inoculation and soon after that the root growth was rapid. NAA has more effective than IBA on induction of rooting as days required to rooting was only 8 to10 days as against the 10 to15 days required for similar response in case of IBA (Shirgurkar *et al*., 2001).

Discussion

The dependence of cultured explants on bud break response and shoot multiplication has already been established and extensively discussed (Babu *et al.*,1992). This has also been recently reported in the case of micro propagation of other Zingiberaceae like *curcuma longa* (Balachandran *et al*.,1990), *Zingiber officinale* (Hashim et al. 1998 Sharma and Singh 1994;1995, Bhat *et al*.,1994). In the present study, sprouting rhizome bud of *Curcuma longa* (cv. Ranga) showed significantly higher response in the medium with the combination of BAP (2.0 mg/l) + NAA (0.5mg/l). The quality of shoots and the over all growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recoded when BAP was added alone in the medium. Review of literature indicates that the addition of either NAA or IBA or IAA in the culture medium improved the response in a number of species in terms of shoot growth. It has been reported that *Spathiphyllum floribundam* when cultured on media with BA supplement alone, a limited proliferation of explants with a maximum of average of 1.8 shoots per cultured explants was

observed ,while addition of IAA produced an average number of 11.6 shoots per explant (Ramierez-Magon et al. 2001). Similar observations were reported in *Hovenia dulcis* nodal culture (Echeverrigaray et al. 2001). In our study it was observed that addition of NAA 0.5 mg/l with BAP (2.0mg/l) showed improved response over BAP alone. Some authors also suggested that the combination of BAP and NAA were needed for producing more number of multiple shoots on *Curcuma longa* (Hoque *et al*.,1999, Hosoki *et al.*,1977; Noguchi *et al.*,1998).

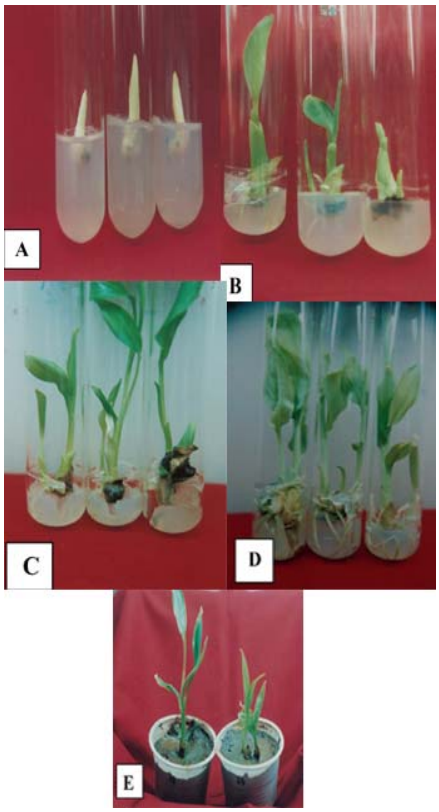

Fig.1:A-E,.Invitropropagation of *Curcuma longa* L. (cv-Ranga)
Fig.A. Rhizome sprouting buds are inoculated in shooting media; Fig. B. Shoot initiation in culture tube;Fig.C. Shoot lets in rooting media
Fig.D. In vitrogenerated shoot lets ready for hardening; g. E. Complete propagule in plastic pot after hardening

Rooting and establishment of plants in soil: Production of plantlets with profuse rooting in *invitro* is important for successful establishment of regenerated plants in soil (Sharma and Singh ,1994 ;1995; Sunitibala et al. 2001).The auxins NAA and IBA were used singly to induce rooting from *invitro* raised shoot lets. A range of concentration was tested (0.25,0.05,1.0,1.5,2.0, 2.5 and 3.0 mg/l) for rooting. In the present study $^{1}/_{2}$ strength MS basal medium and the two different auxins (NAA and IBA) were tried, the maximum results on rooting were obtained on half MS medium with supplementation of NAA (1.5&2.0 mg/l) than IBA (1.5&2.0 mg/l). Our observation are in accordance with the result of Inden et al. (2003) in *Z. officinale* and Salvi et al. (2000, 2001) in turmeric. The well rooted plants were transferred to plastic cups containing vermiculite for hardening and kept under controlled condition (Fig-E). Upon transferred to vermiculite medium plants started producing fresh shoots and roots after one week of transplantation. Later they were transferred to the field and the survival rate was 95% .The efficient micro-propagation technique described here may be highly use full for raising disease free quality planting material of *Curcuma longa* (cv. Ranga) for commercial and off season cultivation which not only help the socioeconomic development of the farmers but also fulfill the spice value and market demand including the conservation of genetic stock of the native species of Orissa for need of the hour.

Conclusion

In the present investigation the *in vitro* micro-plantlet multiplication system of cultivated variety Ranga of *Curcuma longa* has been optimized through rhizome sprouting bud as source of explant. However, our developed protocol can be used to produce a higher amount of large scale propagules as compared to previously reported protocols. Production of *in vitro* plantlets would be a suitable methodology for direct regeneration of shoot lets as a source of disease free quality planting material that could be stored and transported easily and a step forward towards commercial scale of propagule production in turmeric.

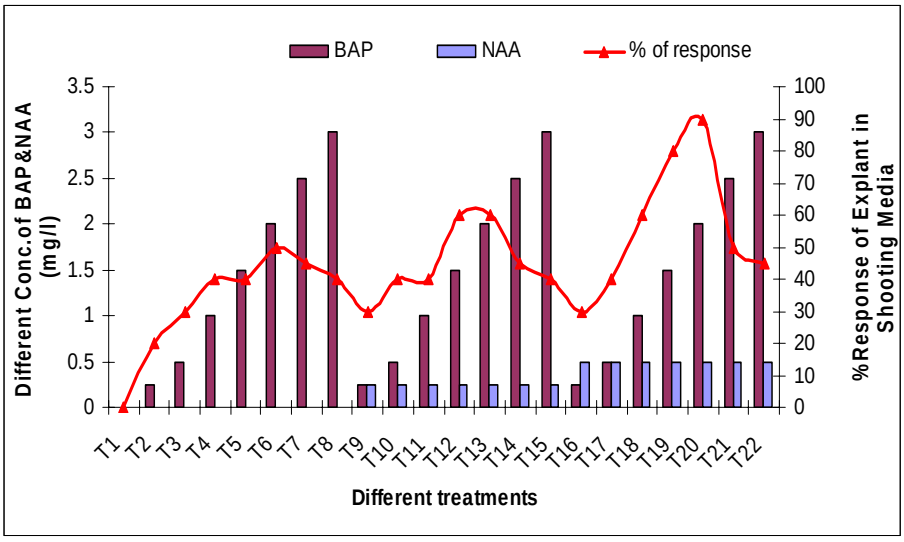

Figure-2A: Shooting Medium: Showing % of Explant Response in Different Treatments of BAP and NAA in MS medium.

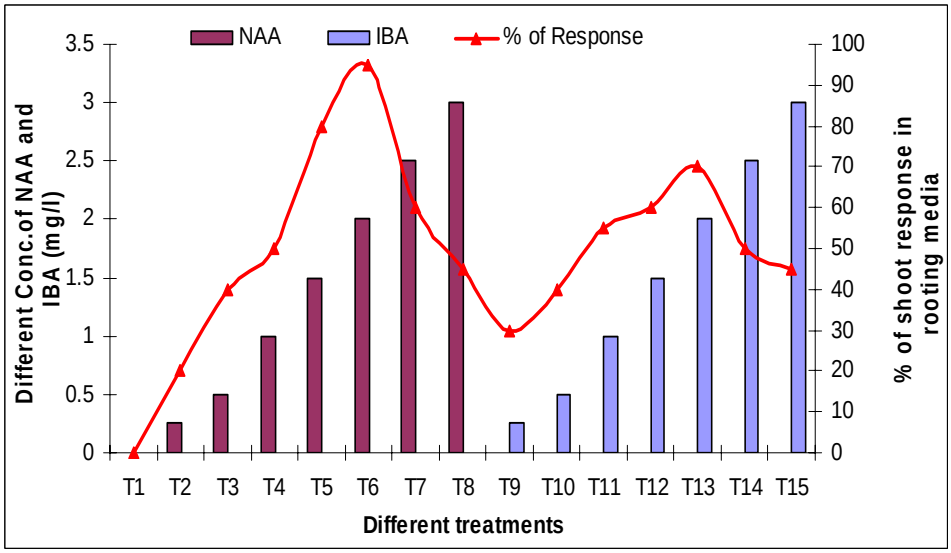

Figure-2B: Rooting Medium: Showing % of Micro shoots Response in Different Treatments of NAA and IBA in ½ MS medium

Table-1: Shoot formation in rhizome sprouting of *Curcuma longa* L. (cv.Ranga) cultured on semisolid MS medium supplemented with various concentration of BAP and NAA [20 culture tube per treatment, data scored after 6 weeks]

Different treatments	Growth Regulators(mg/l)		% of Explant response	Days to bud break	Mean No of shoot/explant ±S.E.	Mean shoot length(cm) ±S.E.	Mean No of nodes/shoot ±S.E.
	BAP	NAA					
T1	0	0	-	-	-	-	-
T2	0.25	0	20	12-15	2.0 ± 0.17+	1.1 ± 0.14+	1.0 ± 0.09+
T3	0.5	0	30	12-15	2.5 ± 0.04+	1.6 ± 0.08+	1.6 ± 0.20+
T4	1.0	0	40	12-15	2.5 ± 0.23+	2.0 ± 0.14+	2.1 ± 0.14+
T5	1.5	0	40	12-15	2.6 ± 0.29	2.6 ± 0.09	2.0 ± 0.20
T6	2.0	0	50	12-15	2.8 ± 0.14	2.6 ± 0.04	2.3 ± 0.22
T7	2.5	0	45	14-16	2.2 ± 0.26+	2.8 ± 0.08+	2.2 ± 0.16+
T8	3.0	0	40	14-16	2.0 ± 0.18+	2.5 ± 0.33+	2.0 ± 0.24+
T9	0.25	0.25	30	12-15	2.2 ± 0.09+	2.5 ± 0.12+	1.5 ± 0.09+
T10	0.5	0.25	40	12-15	2.7 ± 0.04+	2.8 ± 0.24+	2.3 ± 0.17+
T11	1.0	0.25	40	10-12	3.1 ± 0.16+	3.1 ± 0.32+	2.0 ± 0.14+
T12	1.5	0.25	60	10-12	3.3 ± 0.14	3.0 ± 0.36	2.5 ± 0.12
T13	2.0	0.25	60	12-15	3.2 ± 0.32	3.2 ± 0.16	2.8 ± 0.12
T14	2.5	0.25	45	12-15	2 .4 ± 0.30+	2.6 ± 0.24+	2.8 ± 0.21+
T15	3.0	0.25	40	14-16	2.0 ± 0.21+	2.4± 0.34+	2.6 ± 0.32+
T16	0.25	0.5	30	12-15	2.2 ± 0.28+	2.1 ± 0.20+	2.4 ± 0.12+
T17	0.5	0.5	40	12-15	2.3 ± 0.14+	2.2± 0.12+	2.6 ± 0.08+
T18	1.0	0.5	60	10-12	3.5 ± 0.41+	3.1 ± 0.33+	3.1 ± 0.24+
T19	1.5	0.5	80	8-10	4.5 ± 0.12	4.6 ± 0.16	3.5 ± 0.08
T20	2.0	0.5	90	8-10	7.0 ± 0.18	5.4 ± 0.09	4.0 ± 0.28+
T21	2.5	0.5	50	10-12	3.5 ± 0.08+	3.2 ± 0.18+	3.0 ± 0.09+
T22	3.0	0.5	45	10-12	3.2 ± 0.16+	2.4 ± 0.24+	2.6 ± 0.20+

[20 culture tube per treatment; repeated thrice. Means are calculated by Post-Hoc Multiple Comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.:-Standard error of mean]

Table-2:Influence of different levels of NAA and IBA on rooting response of *in vitro* generated shoot lets of *Curcuma longa* L.(cv.Ranga) [20culture tube /treatment ,data scored after 4 weeks]

Different treatments	Growth regulators augmented with1/2 strength MS basal medium(mg/l)		% of Explant Response	Days to root initiation	Mean root numbers ±S.E.	Mean root length (cm) ±S.E.
	NAA	IBA				
T1	0	0	-	-	-	-
T2	0.25	0	20	12-15	1.1 ± 0.12+	1.0 ± 0.09+
T3	0.5	0	40	10-12	2.1 ± 0.12+	2.0 ± 0.09+
T4	1.0	0	50	10-12	2.6 ± 0.09+	2.2 ± 0.20+
T5	1.5	0	80	8-10	5.0 ± 0.20	3.5 ± 0.16
T6	2.0	0	95	8-10	7.3 ± 0.32	4.5 ± 0.12
T7	2.5	0	60	10-12	4.2 ± 0.34+	3.2 ± 0.24+
T8	3.0	0	45	10-12	3.2 ± 0.18+	2.6 ± 0.28+
T9	0	0.25	30	12-15	1.0 ±0.04+	1.5 ± 0.04+
T10	0	0.50	40	12-15	2.3 ± 0.12+	2.3 ± 0.12+
T11	0	1.0	55	10-12	2.4 ± 0.04+	2.3 ± 0.14+
T12	0	1.5	60	10-12	2.5 ± 0.28	2.4 ± 0.18
T13	0	2.0	70	10-12	2.8 ± 0.12	2.6 ± 0.12
T14	0	2.5	50	12-15	2.6 ± 0.16+	2.3 ± 0.20+
T15	0	3.0	45	12-15	2.5 ± 0.32+	2.0 ± 0.09+

[20 culture tube per treatment; repeated thrice. Means are calculated by Post-Hoc Multiple Comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.:-Standard error of mean]

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