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THE EFFECT OF PGRs ON *IN VITRO* DEVELOPMENT OF PROTOCORMS, REGENERATION AND MASS MULTIPLICATION DERIVED FROM IMMATURE SEEDS OF *RHYNCHOSTYLIS RETUSA* (L.) BLUME

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ABSTRACT
Rhynchostylis retusa is a monopodial, epiphytic orchid. Owing to its high commercial value in the floricultural industry, mass propagation provides an alternative means of satisfying the demand. Unfortunately, conventional propagation is slow and difficult, suggesting *in vitro* methods for mass multiplication may be more appropriate. In this case germination ability and early developments of PLBs in MS, ½MS, B_5 and PM media were evaluated. In addition, different plant growth regulators *viz.* BAP, Kin, NAA and IAA were used either single or in combination. The maximum percentage of seed germination (72.60%) was recorded after 7-8 weeks of culture initiation in MS medium. Secondary protocorms were developed from primary PLBs on MS medium fortified with different concentrations and combinations of cytokinins (BAP and Kin) and auxins (NAA, Picloram, IBA and IAA). The highest numbers of secondary PLBs (16.0) were obtained from each of primary protocorms in MS medium supplemented with 1.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA. The maximum root induction (7.0 per shoot) was observed in ½MS medium that supplemented with 1.0 mgl^{-1} IAA. Plants were acclimatized successfully (survive 80%) in the potting mixture containing brick pieces, charcoal, coconut husk in the ratio of 1:1:2 and eventually established under natural condition.

KEY WORDS: Epiphytic orchid, PLBs, Regeneration, Secondary protocorms.

INTRODUCTION

Orchids are important in the floriculture industry due to beautiful foliage, colourful and fragrant flowers of varying shapes, and long vase life of cut flowers and also having medicinal properties (Bhattacharjee *et al*., 2015). This group of plants is valued highly both in the national as well as in the international markets (Kumar *et al*., 2002). *Rhynchostylis retusa* is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence. It is an endangered orchid species that grows in moist areas and blossoms during monsoon. This plant having a robust stem of about 25 cm long and its leaves are strap-shaped and about 25 cm long and the pendulous inflorescences are about 60 cm long which densely flowered. Flowering sits in summer to autumn, especially in May - June (Sinha and Jahan, 2012). The plant is found in semi-deciduous and deciduous dry lowland forests woodlands at elevations of sea level to 1200m (3900 ft.). This orchid is found in Bangladesh, Benin, Burma, Cambodia, China, India, Indonesia, Laos, Malaysia, Nepal, Philippines, Singapore, Sri Lanka, Thailand and Vietnam (Chowdhury *et al*., 2014). The whole plant preparations of *Rhynchostylis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations (Kumar *et al*., 2012). It is also used as an emollient and in the treatment of throat inflammation (Shanavaskhan *et al*., 2012). The plant leaf juice and aerial roots are also used in ear pain and cleaning (Basumatary *et al*., 2004). In the Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das *et al*., 2012). *Rhynchostylis retusa* roots are also used to cure malarial fever (Tiwari *et al*., 2012, Radhika *et al.*, 2013). Juice of roots applied to cuts and wounds. Dried flowers are used as insect repellent and to induce vomiting (Subedi *et al.*, 2013). It is also reported that the plant showed significant antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Hossain, 2011). Many attractive *Rhynchostylis* orchids have become commercially important in potted plant industries. These species needed to be protected from the danger of extermination through deforestation in Bangladesh and also in the world. Therefore, a rapid multiplication in commercial scale by micropropagation of this species is required. The main objective of the study was to develop *in vitro* micropropagation methods for multiplication of this rare orchid and strengthen the resource base through restoration.

MATERIALS & METHODS

Plant Materials

The immature capsule of *Rhynchostylis retusa* was used as seed source which was collected from Rajshahi, Bangladesh (Fig. 1a).

Methods

Sterilization of capsule and inoculation

The capsules were surface sterilized by submerging them in 0.2% (w/v) $HgCl_2$ solution for 10 minutes with occasional agitation followed by a dip in 70% ethanol for 25-30 seconds. The sterilized capsules were washed with sterile distilled water for 5-6 times and then cut them longitudinally with a sterile surgical blade in the laminar

air flow cabinet. The feathery seeds were then taken out with the help of a sterile forceps and were inoculated to the media taken in culture vessels. The lid of culture vessels was closed tightly and finally sealed with parafilm very carefully.

Media and culture condition:

Four basal media *viz.* MS and ½MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968) and PM (Phytamax^{TM}, P-1056, Sigma, USA) amended with 3% sucrose and 0.8% agar were used on germination and for protocorm (PLBs) development. One set of each seed culture was maintained on amended with 2% (w/v) sucrose for each medium. The pH for all media was adjusted at 5.6-5.8. The media was autoclaved at a temperature of 121°C at pressure of 15 psi for 15 minutes. The culture vessels with inoculated seeds were maintained in the culture room under a photo period of 16h light and 8h dark at 25±2°C. Seed germination data were recorded after 50 days of inoculation. Different developmental stages of PLBs (*i.e.* percentage of protocorms with vegetative apex, plantlets with 2-3 leaves) were recorded in every 30 days of culture initiation.

Plant growth regulators

For secondary protocorm development different concentrations of 6-benzylaminopurine (BAP), kinetin (KIN), α-naphthalene acetic acid (NAA), Picloram, indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were used in MS medium. Four concentration of plant growth regulators (0.5, 1.0, 1.5, 2.0 mgl^{-1}) in combination were used for this study to know about their effectiveness in promoting proliferation of protocorms and subsequent plantlet development. Sixty days old primary protocorms (one protocorm per test tube) were used for the production of secondary protocorm and its subsequent developments. Data were recorded on the basis of plantlet development after 60 days of culture initiation. Means of 20 test tubes were taken for each treatment and all experiments were repeated three times.

Rooting and acclimatization

For root induction, six month old plantlets without roots were cultured on half-strength of MS medium supplemented with different concentrations of IAA and/or NAA (0.5, 1.0, 1.5 mgl^{-1}). The well developed rooted plantlets were hardened successfully in the pot containing different mixture of compost *viz.*, (i) brick pieces, charcoal, decaying litter (1:1:1) and sphagnum moss, (ii) brick pieces, charcoal, decaying litter and saw dust (1:1:1:1) and sphagnum moss, (iii) brick pieces, charcoal, coconut husk (1:1:1) and (iv) brick pieces, charcoal, coconut husk (1:1:2) followed by the methods of Das *et al.* (2007). The plantlets were watered alternately in the evening and sprayed with MS nutrient solution (diluted 10 times) fortnightly for about a month.

Data recording and statistical analysis

Data about PLBs, its subsequent regeneration, shoot and root development was recorded. Each value represents an average of 20 replicates and each experiment was repeated three times. Data were subjected to analysis of variance and means were separated by Duncan's multiple range test (DMRT).

RESULTS

Seed germination and protocorm formation

In the present experiment, immature green pods were taken for *in vitro* culture and four types of basal media (MS, ½MS, PM and B5) without PGRs were used to assess their effect on seed germination and protocorm formation. Percentage of seed germination was highest in MS (72.60%) followed by ½MS (60.60%), B_5 (55.20%) and PM (44.40%) (Table 1). Germination was marked by swelling and emergence of the embryo from the testa. Seed germination was first evident by swelling and within 7-8 weeks the undifferentiated embryos formed an irregular shaped cell mass as spherules. After 1-2 weeks, these spherules turned green and formed round structures as protocorms (Fig. 1b). Protocorms became visible after 11 weeks of culture initiation and showed at the vegetative apex (Fig. 1c). This was followed by the development of 2-3 leaf primordia when MS basal medium or half strength of MS medium or B_5 media were used (Fig. 1d). Out of four media MS showed most effective on the development of leaf primordia than others (Table 1). Protocorms cultured in PM medium did not develop beyond the vegetative apex stage, while half-strength of MS and B_5 supported only poor growth of protocorms in comparison with MS medium (Table 1).

TABLE 1: Comparative effect of four culture media on germination of seeds and development of protocorm in *Rhynchostylis retusa*

Media	Time required (weeks)		% of seed germination	Protocorm development (% response)	
	Spherule formation	Protocorm formation	(M ± S.E)	Stage-1	Stage-2
MS	7-8	9-10	72.60 ± 0.50	50.60 ± 0.67	39.40± 0.77
½MS	7-8	9-10	60.60 ± 0.98	40.40 ± 0.94	33.20± 0.92
B_5	8-9	10-11	55.20± 0.84	34.60 ± 0.67	30.40± 0.70
PM	8-9	10-11	44.40 ± 0.79	25.00 ± 0.58	-

MS = Murashige and Skoog (1962); PM = Phytamax^{TM}, P-1056, Sigma, USA; B_5 = Gamborg *et al.* (1968); – = no response. Stage-1: vegetative apex stage and Stage- 2: 2–3 leaves stage.

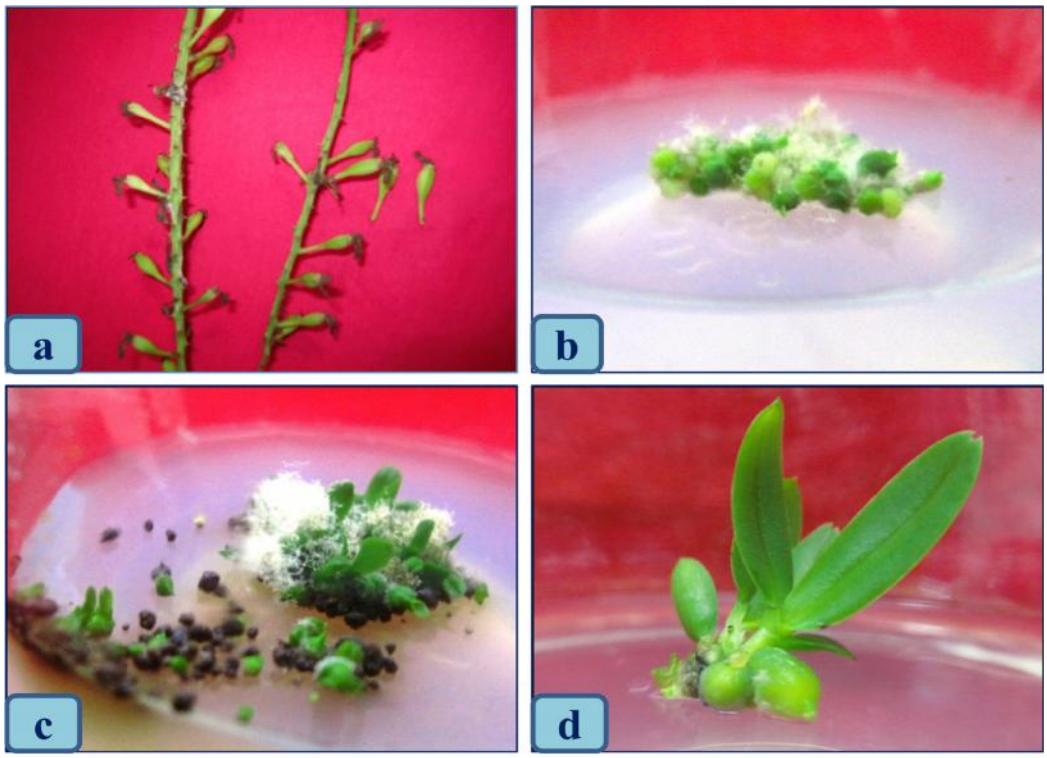

FIGURE 1: Seed germination stages of *R. retusa*. (a) Immature seeds, (b) Rounded protocorms after 8 weeks of culture, (c) Young protocorm with a vegetative apex at the upper side and (d) Complete plantlet with 2-3 leaves and 1-2 roots.

Production of secondary protocorms from primary PLBs

To assess the effectiveness of auxin and cytokinin for protocorm multiplication, 60 days old primary protocorms were sub-cultured on fresh MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0 mgl^{-1}), Kinetin (0.5, 1.0, 1.5, 2.0 mgl^{-1}), NAA (0.5, 1.0, 1.5, 2.0 mgl^{-1}), Picloram (0.5, 1.0, 1.5, 2.0 mgl^{-1}), IBA (0.5, 1.0, 1.5, 2.0 mgl^{-1}) and IAA (0.0, 0.5, 1.0, 1.5 mgl^{-1}) alone and in combination.

TABLE 2: Production of secondary protocorms in *Rhynchostylis retusa* after 60 days of culture initiation in MS medium supplemented with different plant growth regulators (PGRs).

PGR's	Conc. (mgl^{-1})	No. of secondary protocorms M ± S.E
BAP + NAA	0.5 + 0.5	$12.86^{b} \pm 0.23$
	1.0 + 1.0	$16.00^{a} \pm 0.41$
	1.5 + 1.5	$10.60^{cd} \pm 0.23$
	2.0 + 2.0	$10.20^{cd} \pm 0.38$
BAP + IAA	0.5 + 0.5	$10.40^{a} \pm 0.28$
	1.0 + 1.0	$7.20^{b} \pm 0.34$
	1.5 + 1.5	$5.00^{cd} \pm 0.41$
	2.0 + 2.0	$4.00^{cd} \pm 0.18$
BAP + IBA	0.5 + 0.5	$9.20^{a} \pm 0.39$
	1.0 + 1.0	$8.40^{a} \pm 0.26$
	1.5 + 1.5	$8.00^{a} \pm 0.15$
	2.0 + 2.0	$7.20^{b} \pm 0.15$
BAP + Kinetin	0.5 + 0.5	$8.20^{b} \pm 0.28$
	1.0 + 1.0	$4.40^{a} \pm 0.67$
	1.5 + 1.5	$3.80^{a} \pm 0.42$
	2.0 + 2.0	$3.40^{a} \pm 0.29$
BAP + Picloram	0.5 + 0.5	$10.40^{ab} \pm 0.67$
	1.0 + 1.0	$10.00^{a} \pm 0.37$
	1.5 + 1.5	$8.60^{bc} \pm 0.35$
	2.0 + 2.0	$6.40^{c} \pm 0.67$

Secondary protocorms developed from each primary PLBs. Means followed by a common letter are not significantly different at 5% level.

This resulted in the generation of secondary protocorms (PLBs) instead of shoot formation directly (Fig. 2a and b). Protocorm like bodies development initiated after 30 days of culture (Fig. 2a). The maximum number of PLBs (16.00 ± 0.41) per protocorm was observed after 60 days of culture in MS medium that contained BAP (1.0 mgl^{-1}) and NAA (1.0 mgl^{-1}), followed by BAP (0.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) (Table 2). Almost all the PLBs were converted into plantlets (Fig. 2c and d) in the following 20-30 days of the time period, giving rise to multiple numbers of shoots on the same media.

FIGURE 2: Development of plants through PLBs of *Rhynchostylis retusa*. (a) Secondary protocorms (PLBs) developed from a single primary protocorms, (b) Shoot formation from secondary protocorm, (c) Plantlets developed from PLBs, (d) Plants with good shoots and roots, (e) Well-rooted plants ready for hardening, (f) Hardened plants in pots.

Rooting and acclimatization

For increasing the number of roots and their length, plantlets were supplied with different concentration of auxins (Fig. 3 and 4). Half strength of MS medium supplemented with 1.0 mgl^{-1} IAA induced the most roots (7.00) per shoot (Fig. 2e and 3), followed by 1.5 mgl^{-1} IAA. In comparison, NAA was less effective for root formation and promoting root extension. A significant difference was found on root length at different concentrations and combinations of growth regulators. The maximum root length (3.4 cm) was obtained ½ MS medium that supplemented with 1.0 mgl^{-1} IAA followed by 1.0 mgl^{-1} NAA (Fig. 4). The different composts used for hardening of *in vitro* grown plantlets of *R. retusa* were found to be satisfactory for survivability and normal growth of the plantlets. The highest percentage of survivability (80%) with maximum length (5.00 cm) of *R. retusa* hardened plants was obtained on substratum containing brick pieces, charcoal, coconut husk (1:1:2) with a layer of moss (Fig. 2f and Table 3).

TABLE 3: *Ex vitro* establishment of *Rhynchostylis retusa* plantlets.

Substratum of compost	Survival (%)	Height (cm)
Brick pieces + charcoal + decaying litter (1:1:1) + moss (10gm/pot)	69.20 ± 0.80	2.84 ± 0.07
Brick pieces + charcoal + decaying litter + saw dust (1:1:1:1) + moss (10gm/pot)	53.80 ± 0.86	4.02 ± 0.18
Brick pieces + charcoal + coconut husk (1:1:1)	72.00 ± 0.80	2.98 ± 0.10
Brick pieces + charcoal + coconut husk (1:1:2)	80.00 ± 0.82	5.00 ± 0.04

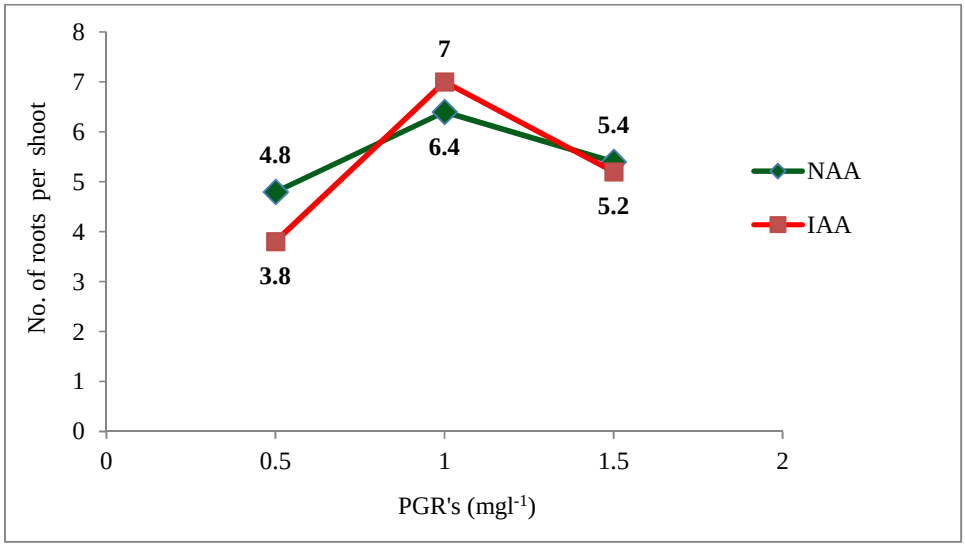

FIGURE 3: Effects of different concentrations of NAA and IAA with ½MS medium on root induction per shoot of *Rhynchostylis retusa.*

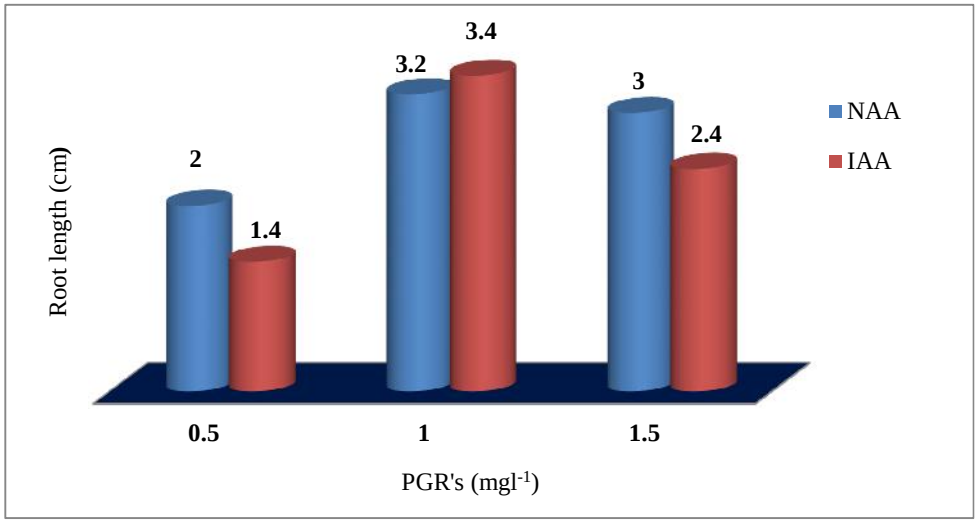

FIGURE. 4 : Effects of different concentrations of NAA and IAA supplemented with ½ MS medium on increase of root length of *Rhynchostylis retusa*.

DISCUSSION

Germination and seedling development in orchids are strikingly different from other flowering plants. A single orchid capsule is estimated to contain millions of seeds, which lack endosperm. In spite of a very large number, only few seeds germinate in nature. Currently the horticultural trade depends on wild orchid population as a source of stock plants, but most are not propagated properly. Nutrient requirements for orchid seed germination are thought to be species specific (Arditti and Ernst, 1984; Kauth *et al*., 2008). It was therefore, not surprising that the various basal media used in the present study, with their different compositions and concentrations of mineral salts, organic supplements and vitamins, varied in their suitability for *in vitro* germination. In the present study found that the MS medium promoted seed germination. Nitrogen is very essential component for plant growth and its source has been shown to have an effect on the germination of different orchid species are reported by Stewart *et al*. (2006). The MS medium contains nitrogen and ammonium stimulated seed germination (Popova *et al*., 2003). Under this study for *Rhynchostylis retusa* we observed that seed germination and PLB's formation was higher also in MS medium. The results are in agreement with the findings of Bhattacharjee and Islam (2015). On contrary, Hossain (2008) reported that 75% of the *Eulophia ibaguense* seeds germinated on MS medium supplemented with 1 mgl^{-1} BAP, but with increasing concentrations of BAP, the seed germination percentage has been declined. In case of *Cypripedium* sp. as terrestrial orchid BAP enhanced seed germination (Depauw *et al*., 1995). The present study showed that primary protocorms responded significantly higher in combination with 1.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA. Highest yield of PLBs obtained in *C. lapine* by Fujii *et al*. (1999) and they suggested that a synergistic effect was also observed in the case of *Cymbidium nativity*. Kusumoto (1978) suggested that the negative effect of

NAA on PLBs proliferation in case of *Cymbidiums* whereas using BAP in the medium showed positive effect on protocorm formation. In case of rooting, IAA was found to be the most superior auxin for promoting root formation and increase of root length. The maximum number (7.00) of roots per shoot was recorded in the medium supplemented with 1.0 mgl^{-1} IAA. Sunitibala and Kishor (2009) also reported that about the positive effects with IAA in the case of *Dendrobium transparens*. On contrary, Bhadra *et al*. (2002) reported that ½MS + 1.5% (w/v) sucrose without auxin showed better performance in case of root formation and increase of root length in *Dendrobium aphyllum*. However, the highest percentage of survivability of hardened plants showed in the compost of brick pieces, charcoal, decaying litter (1:1:1) with a layer of sphagnum moss. The layer of moss on top proved to be beneficial due to higher retention of moisture content. As reported earlier by Kumaria and Tandon (1994) in *D. fimbriatum* var. *oculatum*, feeding the plantlets with dilute MS nutrient salt solution was found beneficial to the developing hardened plantlets of *C. devonianum*. This study gives an effective protocol for seed germination, protocorm development and plantlet regeneration from immature seeds which can be used for establishing *Rhynchostylis retusa* populations in Bangladesh and elsewhere.

CONCLUSION

The efficient induction of PLBs and their proliferation from protocorms of *Rhynchostylis retusa* was achieved for large scale propagation. It was observed that MS medium was the best for seed germination and PLBs formation of *Rhynchostylis retusa* orchid. Similarly MS medium supplemented with BAP (1.0 mgl^{-1}) and NAA (1.0 mgl^{-1}) was found to be the most suitable for protocorm development. On the other hand, IAA was the most effective phytohormone for promoting root formation and increase of root length. Thus, the results clearly demonstrated that above culture condition was the most effective for mass multiplication of *Rhynchostylis retusa* and this protocol will be useful to commercial growers for mass multiplication. For further research on biochemical compound isolation for medicine, genetic transformation and conservation of endangered orchids this techniques and protocol will be helpful for researcher.

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