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THE ROLE OF CYTOKININ AND AUXIN IN ORGANOGENESIS OF *PASSIFLORA MOLLISSIMA* AND EVALUATION OF BIOCHEMICAL CHANGES USING ISOZYME AND PROTEIN PROFILES

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Abstract: *Passiflora mollissima* (H.B.K) Bailey is a climber noted specially for its ornamental and medicinal property. It has been micropropagated successfully by culturing nodal and internodal segments. Murashige and Skoog's basal medium with 3% sucrose and augmented with 6-benzyl amino purine at 4.44 µm showed the maximum number (12.5 ± 0.25) of shoot proliferation from the nodal segments. Maximum percentage (82 ± 0.02) of shoot proliferation was also achieved on Murashige and Skoog's basal medium with 3% sucrose and fortified with 4.44 µm 6-benzyl amino purine. Maximum percentage (78.7 ± 0.5) of callus formation was achieved on Murashige and Skoog's basal medium supplemented with 3% sucrose and 4.52 µm 2, 4-dichlorophenoxy acetic acid. Maximum percentage (78 ± 0.02) of shoot proliferation from the internodal derived calli was observed on Murashige and Skoog's medium augmented with 3% sucrose and 4.44 µm 6-benzyl amino purine in combination with 2.69 µm naphthalene acetic acid. The *in vitro* raised shootlets were transferred to half strength Murashige and Skoog's medium supplemented with auxin for rooting. Maximum frequency (83 ± 0.1) of rootlets and maximum number of rootlets (9.2 ± 0.01) per shootlet formation were obtained in half strength Murashige and Skoog's medium fortified with 4.92 µm indole 3-butyric acid. The micropropagated plantlets' genetic uniformity was confirmed through the isozyme and protein profiles. Electrophoretic studies confirmed the genetic similarity between the mother plant and the plant derived from nodal segments; the genetic variation was noticed in calli-mediated plants. The *in vitro* raised plants were hardened in poly-cups. About 80% of plants were established well in the poly-cup stage, and 75% of plants succeeded in the field establishment.

Keywords: *Passiflora mollissima*, *In vitro* shoot proliferation, Callus.

Abbreviations: MS – Murashige and Skoog's medium, PGRs – Plant Growth Regulators, BAP – Benzyl -6- Amino Purine, IAA – Indole -3-Acetic Acid, IBA – Indole Butyric Acid, NAA – Naphthalene Acetic Acid, 2, 4 – D – 2, 4 – Dichlorophenoxy acetic acid, 2, 4, 5 – T - 2, 4, 5 – Tri chloro phenoxy acetic acid,

Introduction

Passiflora mollissima (H.B.K) Bailey climber belongs to the family Passifloraceae, and is distributed in many tropical countries. The plant is cultivated for ornamental purposes and its fruits have medicinal value. Its force is exerted chiefly upon the nervous system, spasmodic disorders, insomnia of infants and old people, control over childhood spasms, whether from dentition, worms or indigestion, cough etc (Yoganarashiman, 1996; Dawit Abebe *et al.*, 2003). Owing to over-exploitation for commercial purposes, viz. ornamental and medicinal, there is a need to develop new protocol for efficient propagation. The major constraints in conventional propagation through seed and stem cutting are poor seed setting and germination percentage. In addition, stem propagation through cuttings was also not satisfactory. The *in vitro* multiplication techniques offer an alternative tool for the large-scale multiplication and conservation. *De novo* regeneration *in vitro* has been reported for a growing list of medicinal, aromatic, economically important trees and horticultural plants by Sivasubramanian *et al.*, (2002) in *Plectranthus vetiveroides*, Singh and Sudarsan, (2003) in *Baliospernmum* sp., Beena *et al.*, (2003) in *Ceropegia cendelobrum*, Martin, (2003) in *Rotula aquatica*; Gupta *et al.*, (2002) in *Pelargonium graveolnes*; Johnson *et al.*, (2002) and Johnson *et al.*, (2005) in *Rhinacanthus nasutus*, and Pawar *et al.*, (2002) in *Solanum surattence*. With this background the present study was initiated to multiply the medicinally and horticulturally important plant using nodal and internodal segments as explants. This technique may rapidly increase the number of propagules for cultivation as well as aid the replacement of natural populations. In addition, it will promote sustained supply and allow the scientific research on the biochemistry and medicinal efficacy of *Passiflora mollissima*. Therefore, the present study was aimed to produce an efficient protocol for large-scale multiplication of the economically important plant and emphasize on *in vitro* organogenesis.

Material and Methods

Mother plants of *Passiflora mollissima* (H.B.K) Bailey were collected from wild and established in the herbal garden of Muthayammal college of Arts & Science as the

source of explants. The top shoot cuttings were separated from the mother plants, defoliated and cut into 3-5cm length and washed with tween-20, then washed with distilled water and surface-sterilized using an aqueous solution of 0.1% (w/v) mercuric chloride for 60 sec and rinsed thrice in distilled water, then cut into 1cm length. For *in vitro* propagation the surface-sterilized shoot tip, nodal and inter-nodal explants were implanted vertically and horizontally onto the Murashige and Skoog (1962) basal solid medium supplemented with 3% sucrose and 0.6% (w/v) agar (Himedia Mumbai). Different concentrations and combinations of plant growth regulators, viz. BAP, Kin, NAA, 2, 4-D and IAA were used for direct and indirect organogenesis. The pH of the medium was adjusted to 5.8 before adding agar and autoclaved at 121° C for 15 min. The cultures were incubated at 25 ± 2°C under cool fluorescent light (2000 lux, 16 h photoperiod)

The callus derived from internodes was sub-cultured onto MS medium supplemented with PGRs such as BAP, Kin, NAA, etc at different concentrations and combinations for shoot regeneration. The *in vitro* raised shootlets (4-5cm long) were excised and transferred to half strength MS medium augmented with different concentrations and combinations of IBA and IAA for root formation. The *in vitro* raised plantlets were hardened in poly-cups containing a mixture of garden soil and sand and was irrigated with 10-times diluted MS basal liquid medium. After 30 days the plants were transferred to the field. The percentage of establishment was observed periodically.

The samples of leaves from *in vitro* raised shootlets, mother plant and callus were collected. The leaves were washed and ground in a pre-chilled pestle and mortar into a fine paste by adding 500 ml of 1M phosphate buffer of pH 7.0. The ground paste was centrifuged for 10 min in a refrigerated centrifuge at 10,000 rpm. The supernatant (extract) was taken as the protein extract and was stored in Eppendorf tubes and subsequently used for isoenzyme (peroxidase) and protein analysis. For peroxidase isozyme, the PAGE electrophoresis was performed by Anbalagan method (1999). After running, the gel was stained with 0.1% O-Dianisidine acetate buffer (pH 4.2) and 30% hydrogen peroxide and incubated in dark for half an hour.

Then the gel was fixed in 7% acetic acid for 15 min. (Sadasivam and Manickam. 1992). The peroxidase enzymes appeared as dark orange band. Based on the banding profile the Rf values were calculated. The banding profile of isoperoxidase demonstrated the similarity and variation between the mother plant and *in vitro* derived plants.

Results and Discussion

In many cases, explant-source determines the relative success of *in vitro* culture. In the present study also the young explants showed maximum frequency of regeneration compared to the matured ones. Young explants started to proliferate shoot buds three days after culture. The number of shoots produced per explant varied with the concentration and combination of growth regulators used in the medium. The role of BAP in stimulating multiple shoot formation in many plants has been studied extensively (Emmanuel *et al.*,, 2000; Venkateswarlu *et al.*,, 2000; Rout *et al.*,, 2001). Reports of the previous workers agree with our results. In the present study also the nodal segments inoculated on MS medium supplemented with BAP (2.22 µm) showed the maximum percentage (82 ± 0.02) of shootlet formation and length of shoot (4.2+0.84) (Fig. 1A, B & C) (Table 1). The combination of Kin and NAA showed inhibitory effect on shoot initiation in tubes inoculated with nodal segments. In *Pelargomium graveolines* also the same result was obtained (Gupta *et al.*, 2002). The decline in shoot number and length together with basal callusing observed with increasing BAP concentration was also reported in certain other species (Emmanuel *et al.*, 2000).

White friable callus was obtained from the inter-nodal segment inoculated onto the MS medium supplemented with 2, 4-D. The concentration of 2, 4 – D influenced the callus formation, frequency of calli formation and type of calli. The highest frequency (78.7±0.5) of callus formation was observed in MS medium augmented with 2, 4 –D (4.52µm). Similar report was observed by Manickam *et al.*, (2000) on *Withania somnifera*, where callus proliferation declined at lower concentration of 2, 4-D (2.26µm) and only small amount of hard calli were obtained at higher concentration (12.30µm). Calli obtained from the inter-nodal segments were sub-cultured on MS

medium supplemented with cytokinins and auxins for shoot regeneration. That BAP is superior over Kinetin in inducing caulogenesis from callus has been well documented by several workers such as Sarason *et al.*, (1993) in *Piper longum* and Dobos *et al.*, (1994) in *Aristolochia bracteolata*. In the present study also MS medium augmented with BAP (4.44 µm) combined with NAA (2.69 µm) showed the maximum percentage (78±0.02) of shootlet-formation (Table 1). The very same concentration induced the maximum length of shootlets also. Similar observations were reported by Bhattacharya and Bhattacharya (1997) in *Jasminium officinale*.

The *in vitro* raised shootlets through direct and indirect organogenesis were transferred to half strength MS basal medium containing IBA, IAA and NAA for rootlet-formation (Fig. 1D). The highest percentage of rootlets was observed on ½ MS medium augmented with IBA (4.92µm). The highest number (83.4+0.1) and maximum length of roots were observed on the very same concentrations (Fig. 1E). Johnson and Manickam (2003) in *Baliospermum montanum* obtained maximum number of rootlets on the same concentration. That in most of the studies IBA was used for efficient rooting has been reported in the medicinal plants like *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Hemidesmus indicus* (Sreekumar *et al.*, 2000). These results were similar to those of the present study, as IBA showed maximum number of rootlets. In our studies, IAA, IBA, and NAA promoted the root formation and increased the number of roots on *in vitro* grown shootlets. Level of IBA increase led to a decrease in root production. The ½ MS medium augmented with NAA in combination with IAA also showed rootlet-formation, but maximum results were observed in IBA alone. This indicates that these auxins may be more rapidly metabolized (either by conjugation or oxidation) than the other. This can in part be attributed to the inhibitory effect of ethylene produced by IAA. Interestingly, a combination of IAA and IBA increased rooting in *Ailanthus triphysa* cultures, but combining with auxins decreased rooting in *Stackhousia tryonii*, indicating antagonistic effects (Bhatia *et al.*, 2002). The *in vitro* raised plants were placed in poly-cups for hardening. Nearly 80% (16/ 20) of plants were established in poly-cups

(Fig. 1F). Nearly 75% (12/16) of plants were established in the herbal garden of Muthayammal College of Arts and Science, Rasipuram, Tamil Nadu, India.

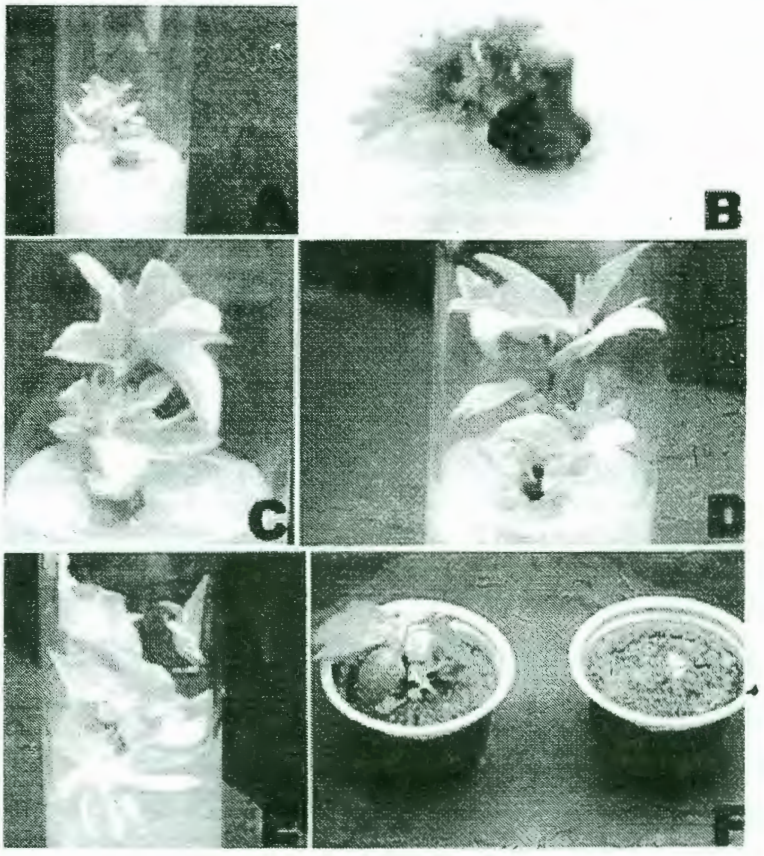


Fig: 1 Micropropagation of Passiflora mollissima (H.B.K) Bailey
A. Initial stage of the shootlets formation after 5 days
B. Seven days *in vitro* raised old shootlets
C. Ten days old *in vitro* raised shootlets
D. *In vitro* raised Plantlets initial stage
E. *In vitro* raised plantlet with well developed shoots and roots
F. Hardening initial stage plantlets in poly cups

The micropropagated plants were genetically confirmed through isoenzyme analysi and SDS-PAGE. The protein patterns were revealed by SDS-PAGE for mothe plant, the *in vitro* grown plantlet directly regenerated using nodal segment and callu mediated plant (at different stage and different tissue). Callus-mediated plants showed the maximum number of bands compared to the mother plant, nodal an shoot tip derived plants (Fig. 2 A). There was no difference between mother and *in vitro* raised plants through direct regeneration by nodal and shoot tip segments (Fig. 2 A). The nodal derived plantlets and mother plants showed 100% similarity index

and no variation between the mother and daughter plants. The isozyme profile also confirmed the similarity between mother and daughter plants (Fig. 2 B). However, the callus mediated plant showed some difference between the mother and nodal derived *in vitro* plants: the protein bands pattern also showed the variation (Fig. 2 A). Our genetic conformity analysis revealed the genetic uniformity and variation. Similar kind of analysis was performed by Mondal *et al.*; (2002), Merce *et al.*, (2003), Johnson (2003) and Johnson *et al.*, (2005). In the present study we evolved a suitable protocol for large-scale multiplication of the ornamental plant *Passiflora mollissima* via direct and indirect regeneration. In addition, we devised the biochemical marker for the mother plant and *in vitro* raised plants. which will help the plant breeders, pharmaceutics and horticulturists. This successful *in vitro* propagation of *Passiflora mollissima* will aid in large-scale asexual propagation of this plant by increasing the number of plants that can be produced per year compared to the traditional methods.

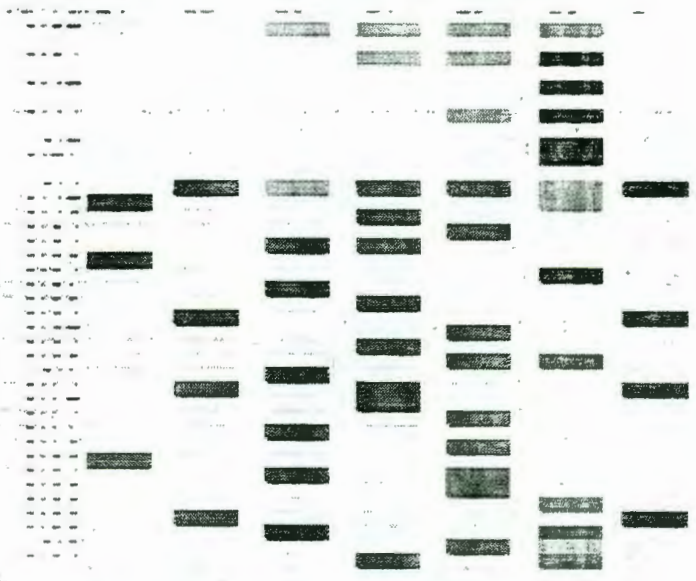


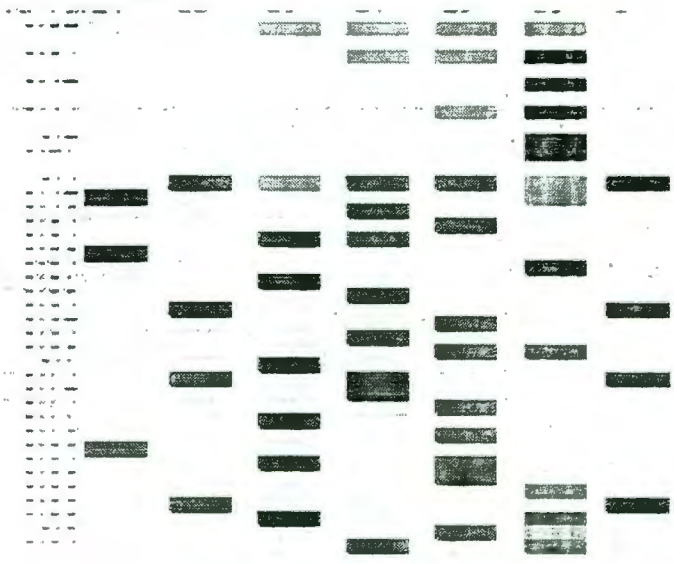
Fig: 2 A. - Histogram of SDS – PAGE Protein Banding pattern of Passiflora mollissima at different Stage

Fig: 2 B. Iso - Peroxidase Profile of in vitro and mother plants of Passiflora mollussim

Table 1: Effect of plant growth regulators on organogenesis of *Passiflora mollissima* (H.B.K) Bailey on MS medium.

MS medium with plant growth regulators (µM)						% of shootlets formation ± S.E	% of callus induction ± S.E	Mean length of shootlets ± S.E	Mean Number of shootlets / explants ± S.E	% of rootlets formation ± S.E	No of rootlets / shootlets ±S.E	% of Rootlets formation ± S.E
BAP	KIN	NAA	2, 4-D	IBA	IAA							
Nodal Segment used as explants												
2.22	0.0	0.0	0.00	0.00	0.00	62 .3 ± 0.05	0.00	4.2 ± 0.84	7.6 ± 0.88	0.00	0.00	0.00
4.44	0.0	0.0	0.00	0.00	0.00	82.2 ± 0.02	0.00	3.4 ± 0.82	12.5 ± 0.25	0.00	0.00	0.00
6.66	0.0	0.0	0.00	0.00	0.00	60 .8 ± 0.12	0.00	2.6 ± 0.1	6.4 ± 0.1	0.00	0.00	0.00
8.88	0.0	0.0	0.00	0.00	0.00	55.6 ± 0.02	0.00	2.5 ± 0.88	4.8 ± 0.02	0.00	0.00	0.00
11.1	0.0	0.0	0.00	0.00	0.00	50.6 ± 0.03	0.00	1.5 ± 0.85	2.35 ± 0.75	0.00	0.00	0.00
0.0	4.65	2.69	0.00	0.00	0.00	45.5 ± 0.01	0.00	3.3 ± 0.01	2.2 ± 0.01	0.00	0.00	0.00
Inter-nodal segments used as explants												
0.00	0.00	0.00	2.26	0.00	0.00	0.00	64.7 ± 0.9	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	4.52	0.00	0.00	0.00	78.7 ± 0.5	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	6.78	0.00	0.00	0.00	60.2 ± 0.5	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	9.04	0.00	0.00	0.00	56.7 ± 0.6	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	12.30	0.00	0.00	0.00	52.4 ± 0.4	0.00	0.00	0.00	0.00	0.00
***In vitro* derived calli used as explants**												
4.44	0.0	0.0	0.00	0.00	0.0	68.3 ± 0.05	0.00	2.4 ± 0.01	6.2 ± 0.34	0.00	0.00	0.00
4.44	0.0	2.69	0.00	0.00	0.0	78.8 ± 0.02	0.00	3.4 ± 0.04	7.2 ± 0.03	0.00	0.00	0.00
6.66	0.0	2.69	0.00	0.00	0.0	60.6 ± 0.12	0.00	2.0 ± 0.02	4.2 ± 0.3	0.00	0.00	0.00
4.44	0.0	0.0	0.00	0.00	2.85	70.8 ± 0.02	0.00	1.3 ± 0.01	5.3 ± 0.21	0.00	0.00	0.00
0.0	4.65	0.0	0.00	0.00	2.85	50.6 ± 0.04	0.00	2.5 ± 0.03	1.4 ± 0.17	0.00	0.00	0.00
0.0	4.65	2.69	0.00	0.00	0.0	45.3 ± 0.03		2.2 ± 0.12	3.2 ± 0.34	0.00	0.00	0.00
***In vitro* derived shoot-let used as explants**												
0.0	0.0	0.0	0.0	4.92	0.0	0.0	0.0	0.0	0.0	83.8 ± 0.1	9.2 ± 0.01	8.8 ± 0.7
0.0	0.0	2.69	0.0	0.0	5.71	0.0	0.0	0.0	0.0	70.9 ± 0.02	6.2 ± 0.20	5.2 ± 0.58
0.0	0.0	0.0		9.84	0.0	0.0	0.0	0.0	0.0	78.2 ± 0.01	7.6 ± 0.2	7.8 ± 0.23

and no variation between the mother and daughter plants. The isozyme profile also confirmed the similarity between mother and daughter plants (Fig. 2 B), However, the callus mediated plant showed some difference between the mother and node derived *in vitro* plants: the protein bands pattern also showed the variation (Fig. 2 A). Our genetic conformity analysis revealed the genetic uniformity and variation. Similar kind of analysis was performed by Mondal *et al.;* (2002), Merce *et al.*, (2003), Johnson (2003) and Johnson *et al.*, (2005) in the present study we evolved a suitable protocol for large-scale multiplication of the ornamental plant *Passiflora mollissima* via direct and indirect regeneration. In addition, we devised the biochemical marker for the mother plant and *in vitro* raised plants. which will help the plant breeders, pharmaceutics and horticulturists. This successful *in vitro* propagation of *Passiflora mollissima* will aid in large-scale asexual propagation of this plant by increasing the number of plants that can be produced per year compared to the traditional methods.



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6.66	0.0	0.0	0.00	0.00	0.00	60 .8 ± 0.12	0.00	2.6 ± 0.1	6.4 ± 0.1	0.00	0.00	0.00
8.88	0.0	0.0	0.00	0.00	0.00	55.6 ± 0.02	0.00	2.5 ± 0.88	4.8 ± 0.02	0.00	0.00	0.00
11.1	0.0	0.0	0.00	0.00	0.00	50.6 ± 0.03	0.00	1.5 ± 0.85	2.35 ± 0.75	0.00	0.00	0.00
0.0	4.65	2.69	0.00	0.00	0.00	45.5 ± 0.01	0.00	3.3 ± 0.01	2.2 ± 0.01	0.00	0.00	0.00
Inter-nodal segments used as explants												
0.00	0.00	0.00	2.26	0.00	0.00	0.00	64.7 ± 0.9	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	4.52	0.00	0.00	0.00	78.7 ± 0.5	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	6.78	0.00	0.00	0.00	60.2 ± 0.5	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	9.04	0.00	0.00	0.00	56.7 ± 0.6	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	12.30	0.00	0.00	0.00	52.4 ± 0.4	0.00	0.00	0.00	0.00	0.00
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6.66	0.0	2.69	0.00	0.00	0.0	60.6 ± 0.12	0.00	2.0 ± 0.02	4.2 ± 0.3	0.00	0.00	0.00
4.44	0.0	0.0	0.00	0.00	2.85	70.8 ± 0.02	0.00	1.3 ± 0.01	5.3 ± 0.21	0.00	0.00	0.00
0.0	4.65	0.0	0.00	0.00	2.85	50.6 ± 0.04	0.00	2.5 ± 0.03	1.4 ± 0.17	0.00	0.00	0.00
0.0	4.65	2.69	0.00	0.00	0.0	45.3 ± 0.03		2.2 ± 0.12	3.2 ± 0.34	0.00	0.00	0.00
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0.0	0.0	2.69	0.0	0.0	5.71	0.0	0.0	0.0	0.0	70.9 ± 0.02	6.2 ± 0.20	5.2 ± 0.58
0.0	0.0	0.0		9.84	0.0	0.0	0.0	0.0	0.0	78.2 ± 0.01	7.6 ± 0.2	7.8 ± 0.23

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