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Research Article

NITROGEN ASSIMILATING ENZYME OF AZOLLA AS INFLUENCED BY POTASSIC FERTILIZER

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A B S T R A C T

Association between the Azolla and *Anabena azollae* resulted in fixation of atmospheric nitrogen through a process of reduction of atmospheric di-nitrogen mediated by the enzymes, glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) both under symbiotic and free-living conditions. The activity of these enzymes is increased by enrichment of nutrients especially K which usually applied to rice crop and improve the biological nitrogen fixation by Azolla. An incubation experiment was conducted by growing *Azollae filiculoides* with the agriculturally important potassic fertilizers (Potassium Chloride, Potassium shulphate) as main plot in seven concentrations (0, 5, 10, 20, 30, 40 and 50 ppm of K) as sub-plots laid down in split-plot design replicated thrice to study the impact of these fertilizer on the activities of nitrogen-fixing enzymes. The activity of glutamate synthase enzyme was predominant in azolla than other enzymes. The maximum activity of glutamate synthase (1.567 n mole min^{-1} mg^{-1}), glutamate dehydrogenase activity (0.158 n mole min^{-1} mg^{-1}) and glutamine synthatase (0.908 n mole min^{-1} mg^{-1}) was observed under the 40 ppm K solution especially on 30th day after incubation. The potassium sulphate fertilizer was superior than the potassium chloride in influencing the growth and maintaining higher enzyme activity in azolla.

INTRODUCTION

Water fern Azolla fixes atmospheric nitrogen in association with nitrogen-fixing cynobacterium *Anabbaena azollae* and thereby serves as an effective biofertilizer in agriculture (Neirzwicki-Bauer, 1990 and Peters 1991). Azolla can be cultured in nutrient enriched water without significantly influencing their nitrogen fixation rates (Reddy, 1987). The response of Azolla to potassic fertilization is most effective in stimulating the growth of Azolla (Kannaiyan, 1990). A. pinnata - *A. azollae* association grew significantly better in the presence of nitrate as KNO_3 (Rai and Rai, 2003). The ammonia assimilation is mediated by the enzyme glutamine synthatese (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) both under symbiotic and free-living conditions (Yates and Eady, 1989). The GDH appears to be involved in NH_4^+ assimilation when the organisms grown at high NH_4^+ concentration but under N-limited conditions, the GS-GOGAT pathway predominate in N fixation. Further, the enrichment of nutrients especially K which is usually apllied to rice crop increases the activity of these enzymes and improve the biological nitrogen fixation by Azolla (Ray *et.al*, 1978).

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Under these circumstances, a research was conducted to assess the enzymes responsible for N fixation under various concentrations of potassic fertilizers commonly used under Indian farming.

MATERIALS AND METHODS

An incubation experiment was conducted by growing *Azollae filiculoides* with 2 agriculturally important potassic fertilizers (Potassium Chloride, Potassium shulphate) as main plot in seven concentrations (0, 5, 10, 20, 30, 40 and 50 ppm of K) as sub-plots laid down in split-plot design replicated thrice. The Azolla fern was grown in a tray with a dimension of 23 x 15x 6 cm^3 filled with 1.5 litres of potassic solutions. The fern was collected on 7th, 15th, 30th, 60th, 90th and 120th day after incubation / culturing, rinsed with distilled water and analysed for various biometric and biochemical parameters. The N assimilating enzyme such as Glutamate dehydrogenase, Glutamate synthase, and Glutamine synthatese were analysed by following standard methods as proposed by Dohertry (1970), Tempest *et al*. (1970) and Pateman (1969) respectively.

Extraction of enzyme

Nitrogen assimilating enzymes such as glutamate synthase and glutamate dehydrogenase were extracted from 1 g of azolla with 5 ml of 100 mM phosphate buffer pH 7.5 containing 1mM disodium EDTA, 1mM dithioerythritol and 1

% polyvinyl pyrrolidone (PVP) and centrifuged at 10,000rpm for 30 minutes at 4°C.

Glutamate synthase

The enzyme extract (0.2 ml) was added with 1 ml of glutamine, 1 ml of 2-oxoglutarate, 1 ml of NADPH and 1.8 ml of Tris-HCl buffer 50 mM at pH 7.6, incubated for 15 – 30 minutes and recorded absorbance at 340 nm in the Spectrophotometer.

Glutamate dehydrogenase

The enzyme extract (0.2 ml) was added with 1 ml of potassium phosphate buffer, 0.3 ml of 2-oxoglutarate, 0.5 ml of NH_4Cl, 0.12 ml of NADH or NADPH and 8 ml of water, incubated for 15 – 30 minutes at 37°C and recorded absorbance at 340 nm in the Spectrophotometer. The amount of NADH or NADPH oxidised per minute per mg of protein was calculated from the molar extinction co-efficient.

Glutamine synthetase

The enxyme was extracted from 1 g of azolla in 5 ml of 50 mM Imidazole acetate buffer pH 7.8 containing 0.5mM EDTA, 1mM dithioerythritol, 2 mM $MnCl_2$, 0.5 % Glycerol and centrifuged at 10,000 rpm for 30 minutes at 4°C.

The enzyme extract (0.2 ml) was added with 2 ml of L-glutamine, 0.5 ml of sodium arsenate, 0.3 ml of 2 mM $MnCl_2$, 0.5 ml of hydroxylamine, 0.5 ml of ADP and 2 ml of 20 mM Tris-HCl buffer, incubated for 30 minutes at 37°C and recorded absorbance at 340 nm in the Spectrophotometer after adding 1 ml of ferric chloride.

Statistical analysis

The data were analysed statistically as per the procedures given by Panse and Sukhatme (1967) and the treatments were compared based on the critical difference for its effectiveness.

Glutamate synthase (GS-GOGAT)

The enzyme glutamate synthase dominates the nitrogen assimilation in Azolla than other enzymes. All the potassic fertilizers were effective in activating glutamate synthase and mean enzyme content of 0.788 and 0.957 n mole min^{-1} mg^{-1} was observed under potassium chloride and potassium sulphate respectively (Table 1a). Among the K fertilizer, the potassium sulphate was superior in maintaining higher enzyme content during all the days of incubation due to its sulphur bearing nature. The glutamate synthase activity was enhanced with the advancement of period of incubation upto 30th day after incubation and decreased after that. The highest glutamate synthase of 1.249 and 0.966 n mole min^{-1} mg^{-1} was registered on 30th day after incubation by potassium sulphate and potassium chloride respectively. The potassium sulphate fertilizer was superior in maintaining highest glutamate synthase content during all the days after incubation.

The potassium concentration also significantly influenced the glutamate synthase activity during all the days after incubation. In general, the content of glutamate synthase enzyme varied between 0.640 and 1.148 n mole min^{-1} mg^{-1} (Table 1a). The highest mean enzyme content of 1.148 n mole min^{-1} mg^{-1} was registered by the 40 ppm K. As that of fertilizers, the glutamate synthase activity was enhanced with the advancement of period of incubation upto 30th day after incubation and decreased thereafter. The maximum glutamate synthase content of 1.567 n mole min^{-1} mg^{-1} was recorded by the 40 ppm K solution on 30th day after incubation followed by the same concentration on 60th day after incubation (1.264 n mole min^{-1} mg^{-1}). The 40 ppm K registered higher glutamate synthase activity in all the days after incubation followed by 30 ppm K solution except on 15th and 30th day after incubation.

Table 1a Glutamate synthase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the main effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	**15**	**30**	**60**	**90**	**120**	
	Fertilizer						
F_1 (KCl)	0.720	0.748	0.966	0.856	0.802	0.638	**0.788**
F_2 (K_2SO_4)	0.823	1.009	1.249	1.002	0.889	0.757	**0.957**
Mean	**0.771**	**0.884**	**1.108**	**0.929**	**0.846**	**0.697**	
CD (0.05)	0.064	0.147	0.027	0.900	0.015	0.101	
	Concentration						
C_1 (0 ppm)	0.542	0.678	0.848	0.688	0.611	0.472	**0.640**
C_2 (2 ppm)	0.651	0.799	0.993	0.808	0.733	0.568	**0.759**
C_3 (5 ppm)	0.659	0.823	1.034	0.844	0.7757	0.563	**0.780**
C_4 (10 ppm)	0.755	0.868	0.973	0.859	0.825	0.686	**0.828**
C_5 (20 ppm)	0.839	0.898	1.155	0.979	0.906	0.772	**0.925**
C_6 (30 ppm)	0.907	0.930	1.140	0.999	0.965	0.848	**0.973**
C_7 (40 ppm)	0.990	1.086	1.567	1.264	1.077	0.904	**1.148**
C_8 (50 ppm)	0.830	0.947	1.149	0.991	0.893	0.767	**0.928**
Mean	**0.771**	**0.884**	**1.108**	**0.929**	**0.846**	**0.697**	
CD (0.05)	0.061	0.061	0.066	0.0611	0.072	0.069	

RESULTS AND DISCUSSIONS

The ammonia assimilation is mediated by the enzyme glutamine synthatese (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) both under symbiotic and free-living conditions (Yates and Eady, 1989). The effect of K nutrient fertilizer and their concentration on the activities of these enzymes is presented and discussed below.

The interaction between the fertilizer and concentration was significant on 15th, 30th and 60th day after incubation. Irrespective of the period of incubation the highest mean enzyme content of 1.019 and 1.278 n mole min^{-1} mg^{-1} was recorded when the azolla was grown under 40 ppm K solution as potassium chloride and potassium sulphate respectively (Table 1b). Irrespective of the concentration, the highest mean enzyme content of 1.108 n mole min^{-1} mg^{-1} was recorded on 30th day after incubation. The highest enzyme content of

1.267 and 1.868 n mole min^{-1} mg^{-1} was recorded when the azolla incubated under 40 ppm of potassium chloride and potassium sulphate respectively which was 88 per cent and 82 per cent more than the control (0 ppm).

Table 1b Glutamate synthase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the interaction effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	15	30	60	90	120	
F_1C_1	0.477	0.526	0.671	0.599	0.542	0.412	0.538
F_1C_2	0.61	0.642	0.841	0.742	0.687	0.533	0.676
F_1C_3	0.581	0.653	0.906	0.78	0.689	0.472	0.680
F_1C_4	0.687	0.732	0.922	0.827	0.766	0.609	0.757
F_1C_5	0.804	0.779	1.066	0.923	0.891	0.716	0.863
F_1C_6	0.859	0.826	1.028	0.927	0.916	0.803	0.893
F_1C_7	0.934	0.939	1.267	1.103	1.045	0.823	1.019
F_1C_8	0.807	0.866	1.031	0.949	0.882	0.732	0.878
F_2C_1	0.606	0.831	1.025	0.778	0.681	0.531	0.742
F_2C_2	0.691	0.956	1.146	0.874	0.779	0.602	0.841
F_2C_3	0.738	0.994	1.163	0.908	0.824	0.653	0.880
F_2C_4	0.822	1.004	1.025	0.893	0.883	0.761	0.898
F_2C_5	0.874	1.018	1.244	1.035	0.922	0.826	0.987
F_2C_6	0.954	1.135	1.253	1.073	1.014	0.893	1.054
F_2C_7	1.046	1.234	1.868	1.426	1.109	0.983	1.278
F_2C_8	0.852	1.009	1.267	1.034	0.905	0.801	0.978
Mean	0.771	0.884	1.108	0.929	0.846	0.697	
CD (0.05)							
F at C	NS	0.157	0.090	0.113	NS	NS	
C at F	NS	0.085	0.093	0.086	NS	NS	

The observed higher activity of glutamate synthase enzyme than other enzymes was due to its active involvement in GS-GOGAT pathway to incorporate nitrogen in the amide position of glutamine and transferred to α – position of α – keto glutamate in the host azolla as outlined by Boussiba and Gibson (1991). The suppressed enzyme content at the higher concentration was mainly due to its toxicity as observed by Venkataraman and Kannaiyan (1986).

Glutamate dyhydrogenase (GDH)

The enzyme glutamate dehydrogenase content was increased with the advancement of period of incubation in both fertilizers and their concentrations up to 30 days of incubation. In general, the potassium sulphate registered highest mean enzyme content of 0.095 n mole min^{-1} mg^{-1} (Table 2a) followed by the potassium chloride (0.073 n mole min^{-1} mg^{-1}).

The potassium sulphate significantly maintained higher glutamate dehydrogenase content during all the days of incubation and highest content of 0.130 n mole min^{-1} mg^{-1} was observed on 30^{th} day after incubation. The potassium chloride fertilizer also produced highest glutamate dehydrogenase content of 0.116 n mole min^{-1} mg^{-1} on the same 30^{th} day of incubation.

The enzyme content increased with the advancement of period of incubation when the azolla was incubated with various K concentrated solution. In general, the highest mean glutamate dehydrogenase enzyme content of 0.107 n mole min^{-1} mg^{-1} was registered by 40 ppm of K solution followed by 50 ppm with the enzyme content of 0.099 n mole min^{-1} mg^{-1} irrespective of period of incubation (Table 2a). The significantly highest glutamate dehydrogenase activity (0.158 n mole min^{-1} mg^{-1}) was noticed on 30^{th} day after incubation under 40 ppm K followed by 50 ppm in all the days of incubation except 15^{th} and 30^{th} days after incubation in which the 30 ppm K registered next highest enzyme content. It indicates the higher concentration of K restrict the azolla growth by its toxicity. Though the 40 ppm K significantly maintained highest enzyme content during all the days of incubation, which was on par with the next lowest (30 ppm) and next highest (50 ppm) concentration on 15^{th} day after incubation. This shows the insufficient and excess K requirement for azolla growth during these days of incubation.

Interaction between the potassic fertilizer and their concentration significantly influenced glutamate dehydrogeanse enzyme activity during 15^{th} and 30^{th} days after incubation (Table 2b). The average glutamate dehydrogenase content varied between 0.046 and 0.119 n mole min^{-1} mg^{-1}. The highest enzyme content of 0.163 n mole min^{-1} mg^{-1} was maintained by the application potassium sulphate as 40 ppm followed by 30 ppm (0.155 n mole min^{-1} mg^{-1}) which were on par with each other. The same trend was observed during 15^{th} day after incubation. The synthesis of more glutamate dehydrogenase enzyme by the potassium sulphate was mainly due to high water soluble nature of the fertilizer and bearing of S which facilitates higher growth of azolla. Meeks ***et al***. (1985) have reported the same findings. Tilo *et al*. (1989) concluded that potassium dihydrogen phosphate and potassium sulphate were equally effective in increasing the biomass production of Azolla.

Table 2a Glutamate dehydrogenase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the main effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	15	30	60	90	120	
Fertilizer							
F_1 (KCl)	0.038	0.102	0.116	0.077	0.057	0.047	0.073
F_2 (K_2SO_4)	0.068	0.114	0.130	0.100	0.084	0.076	0.095
Mean	0.053	0.108	0.123	0.088	0.071	0.062	
CD (0.05)	0.002	0.009	0.004	0.008	0.002	0.004	
Concentration							
C_1 (0 ppm)	0.040	0.070	0.077	0.059	0.050	0.045	0.057
C_2 (2 ppm)	0.046	0.078	0.097	0.072	0.059	0.053	0.067
C_3 (5 ppm)	0.049	0.100	0.099	0.075	0.062	0.055	0.073
C_4 (10 ppm)	0.052	0.114	0.117	0.085	0.068	0.060	0.082
C_5 (20 ppm)	0.054	0.113	0.134	0.094	0.074	0.064	0.089
C_6 (30 ppm)	0.055	0.130	0.151	0.104	0.079	0.067	0.098
C_7 (40 ppm)	0.069	0.134	0.158	0.113	0.091	0.080	0.107
C_8 (50 ppm)	0.060	0.125	0.150	0.105	0.083	0.071	0.099
Mean	0.053	0.108	0.123	0.088	0.071	0.062	
CD (0.05)	0.003	0.008	0.005	0.007	0.004	0.003	

Table 2b Glutamate dehydrogenase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the interaction effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	15	30	60	90	120	
F_1C_1	0.024	0.068	0.071	0.048	0.036	0.030	0.046
F_1C_2	0.030	0.075	0.084	0.057	0.044	0.037	0.055
F_1C_3	0.035	0.085	0.084	0.060	0.047	0.041	0.059
F_1C_4	0.038	0.111	0.109	0.074	0.056	0.047	0.073
F_1C_5	0.039	0.108	0.129	0.084	0.062	0.050	0.079
F_1C_6	0.040	0.122	0.147	0.094	0.067	0.053	0.087
F_1C_7	0.052	0.126	0.152	0.102	0.077	0.065	0.096
F_1C_8	0.042	0.120	0.148	0.095	0.069	0.055	0.088
F_2C_1	0.056	0.071	0.083	0.070	0.063	0.059	0.067
F_2C_2	0.062	0.081	0.109	0.086	0.074	0.068	0.080
F_2C_3	0.063	0.115	0.114	0.089	0.076	0.069	0.088
F_2C_4	0.065	0.116	0.124	0.095	0.080	0.072	0.092
F_2C_5	0.068	0.117	0.139	0.104	0.086	0.077	0.099
F_2C_6	0.070	0.137	0.155	0.113	0.091	0.081	0.108
F_2C_7	0.085	0.142	0.163	0.124	0.105	0.095	0.119
F_2C_8	0.077	0.130	0.152	0.115	0.096	0.086	0.109
Mean	0.053	0.108	0.123	0.088	0.071	0.062	
CD (0.05)							
F at C	NS	0.013	0.008	NS	NS	NS	
C at F	NS	0.011	0.007	NS	NS	NS	

Glutamine synthetase

The main factor of potassic fertilizer and its concentration significantly influenced the glutamine synthatese content during all the days of incubation except 7th and 30th day. In general, the fertilizer potassium sulphate registered highest mean glutamine synthatese of 0.731 n mole min^{-1} mg^{-1} (Table 3a) followed by potassium chloride (0.671 n mole min^{-1} mg^{-1}). The enzyme activity was more during initial stage of incubation and maintained almost constant value during the later stages of incubation.

Generally, the mean glutamine synthatese content varied between 0.586 and 0.865 n mole min^{-1} mg^{-1} under the interaction effect of fertilizer with its concentration (Table 3b).

Table 3b Glutamine synthatase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the interaction effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	15	30	60	90	120	
F_1C_1	0.504	0.581	0.726	0.615	0.560	0.532	0.586
F_1C_2	0.516	0.588	0.758	0.637	0.577	0.546	0.604
F_1C_3	0.536	0.640	0.769	0.653	0.594	0.565	0.626
F_1C_4	0.552	0.672	0.806	0.679	0.616	0.584	0.652
F_1C_5	0.595	0.677	0.826	0.711	0.653	0.624	0.681
F_1C_6	0.670	0.701	0.833	0.752	0.711	0.690	0.726
F_1C_7	0.742	0.742	0.880	0.811	0.777	0.759	0.785
F_1C_8	0.650	0.680	0.825	0.738	0.694	0.672	0.710
F_2C_1	0.509	0.613	0.723	0.616	0.563	0.536	0.593
F_2C_2	0.570	0.691	0.789	0.680	0.625	0.597	0.659
F_2C_3	0.592	0.804	0.821	0.707	0.649	0.621	0.699
F_2C_4	0.598	0.842	0.862	0.730	0.664	0.631	0.721
F_2C_5	0.614	0.853	0.879	0.747	0.680	0.647	0.737
F_2C_6	0.725	0.863	0.906	0.816	0.770	0.748	0.805
F_2C_7	0.812	0.898	0.936	0.874	0.843	0.828	0.865
F_2C_8	0.703	0.843	0.850	0.777	0.740	0.721	0.772
Mean	**0.618**	**0.731**	**0.824**	**0.721**	**0.670**	**0.644**	
CD (0.05)							
F at C	NS	0.054	NS	NS	0.025	NS	
C at F	NS	0.029	NS	NS	0.026	NS	

Though the significant interaction between the potassic fertilizer and concentrations in maintaining glutamine synthatese content was observed during 15th and 90th day after incubation, the highest glutamine synthatese content of 0.936 n mole min^{-1} mg^{-1} was recorded by the 40 ppm of potassium sulphate on 30th days after incubation.

Table 3a. Glutamine synthatase enzyme (n mole min^{-1} mg^{-1}) in Azolla as influenced by the main effect of fertilizer and their concentration

Treatment	Period of incubation, Days						Mean
	7	15	30	60	90	120	
Fertilizer							
F_1 (KCl)	0.596	0.660	0.803	0.700	0.648	0.622	0.671
F_2 (K_2SO_4)	0.640	0.801	0.846	0.743	0.692	0.666	0.731
Mean	0.618	0.731	0.824	0.721	0.670	0.644	
CD (0.05)	NS	0.051	NS	0.031	0.005	0.038	
Concentration							
C_1 (0 ppm)	0.507	0.597	0.725	0.616	0.562	0.534	0.590
C_2 (2 ppm)	0.543	0.640	0.774	0.659	0.601	0.572	0.631
C_3 (5 ppm)	0.564	0.722	0.795	0.680	0.622	0.593	0.663
C_4 (10 ppm)	0.575	0.757	0.834	0.705	0.640	0.608	0.686
C_5 (20 ppm)	0.605	0.765	0.853	0.729	0.667	0.636	0.709
C_6 (30 ppm)	0.698	0.782	0.870	0.784	0.741	0.719	0.765
C_7 (40 ppm)	0.777	0.820	0.908	0.843	0.810	0.794	0.825
C_8 (50 ppm)	0.677	0.762	0.838	0.758	0.717	0.697	0.741
Mean	0.618	0.731	0.824	0.721	0.670	0.644	
CD (0.05)	0.110	0.021	0.073	0.029	0.018	0.027	

Irrespective of fertilizers used, all the K concentrated solution significantly influenced the glutamine synthatase content during all the days of incubation. The mean enzyme content varied between 0.590 and 0.825 n mole min^{-1} mg^{-1} (Table 3a). Incubation of azolla with 40 ppm K exhibited more glutamine synthatase content during all the days but it was on par with 30 and 50 ppm solution during early stages of incubation. Though the maximum glutamine synthatase content of 0.908 n mole min^{-1} mg^{-1} was observed under 40 ppm potassic solution which were on par with the 20, 30 and 50 ppm on 30th day after incubation

The same concentration significantly maintained highest glutamine synthatase content of 0.898and 0.873 n mole min^{-1} mg^{-1} on 15th and 90th days after incubation respectively.

Both K fertilizers were effective in synthesising the glutamine synthatese enzyme and a typical increase was noticed in potassium sulphate. The 40 ppm K was sufficient for synthesising adequate amount of glutamine synthatese (Pan and Coote,1979). Paters *et al*. (1979) confirmed the host assimilation of nutrients with respect of GS activity and Uheda (1986) observed the higher GS and GDH activity in cavity hairs than leaves in Azolla.

CONCLUSIONS

The activity of glutamate synthase enzyme was predominant in azolla than other enzymes due to an active involvement of GS-GOGAT pathway to incorporate nitrogen in the amide position of glutamine. The maximum activity of glutamate synthase (1.567 n mole min^{-1} mg^{-1}), glutamate dehydrogenase activity (0.158 n mole min^{-1} mg^{-1}) and glutamine synthatase (0.908 n mole min^{-1} mg^{-1}) was observed under the 40 ppm K solution especially on 30^{th} day after incubation. The activity of the enzyme at 40 ppm was on par with 30 and 50 ppm solution during early stages of incubation which implies the insufficient and toxicity of K respectively for azolla. The potassium sulphate fertilizer was superior than the potassium chloride in influencing the growth and maintaining higher enzyme activity in azolla.

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