Polyamine metabolism and lipoxygenase activity during *Fusarium oxysporum* f. sp. *ricini* -Castor interaction

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Published online: 10 March 2013

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Abstract Polyamine oxidase and lipoxygenase enzymes are key players for hyper sensitive reaction (HR) during incompatible interaction of host-pathogen. Thus, the role of lipoxygenase and polyamines was studied in the wilt pathogen infected and non infected tissues of resistant and susceptible genotypes of castor at 0 days after infection (DAI), 5 DAI and 10 DAI (30 days after sowing). The lipoxygenase (LOX) and polyamine oxidase (PAO) activities were higher in the incompatible interaction at all the stages of analysis. The constitutive level of malondialdehyde (MDA) content, a product of lipid peroxidation was higher in susceptible genotypes (VP-1 and VI-9), while induced level was higher in resistant genotypes (48-1 and SKP-84) at 5 DAI and 10 DAI. Polyamine profiling using HPTLC showed higher spermidine and spermine content in resistant genotypes at 10 DAI. Furthermore, spermidine was detected only in the roots of resistant genotypes at 10 DAI. These results suggest the role of high titers of polyamines, LOX and PAO in disease resistance possibly through HR induction.

Keywords Castor · HPTLC · Lipoxygenase · MDA · Polyamines · Wilt

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Introduction

Castor (*Ricinus communis* L.) is the industrially important non edible oilseed crop belongs to family *Euphorbiaceae*. Castor crop is affected by several biotic and abiotic stresses, which affect successful and profitable cultivation of the crop. Among them, diseases play an important role to cause severe yield losses. Castor wilt (*Fusarium oxysporum* f. sp. *ricini*), is a serious problem and causes 80 % yield losses. (Mhaske et al. 2013). Cultivars resistant to this fungus are the most practical way to control this disease. Both compatible and incompatible responses induce alterations in plant metabolism; only in the latter the plant is able to efficiently block pathogen penetration without suffering excessive damage.

After pathogen recognition, a multitude of plant resistance-associated reactions are initiated, such as ion fluxes across plant membranes, the generation of reactive oxygen species (ROS), phosphorylation of specific proteins, activation of cell wall strengthening enzymes, transcriptional activation of several defense related genes, induction of phytoalexins, localized cell death at infection sites (Gupta et al. 2010). One ultimate characteristic of hyper sensitive reaction (HR) is the loss of membrane integrity, and thus HR is often associated by electrolyte leakage and lipid peroxidation accomplished by the enzyme lipoxygenase (LOX). Initiation of HR membrane damage by LOXs has been suggested as an alternative hypothesis to free radical action, and the process might be propagated by autoxidation (Rusterucci et al. 1999).

In addition, polyamine catabolism also contribute to defense responses through two reaction products : γ -aminobutyric acid (GABA), an important metabolite, largely and rapidly produced in response to biotic and abiotic stresses (Cona et al. 2006) and the reactive oxygen species, H_2O_2 , which has long been recognized to play a key role in defense (Gechev et al. 2006). H_2O_2 is a mediator of several physiological events such as programmed cell death,

lignifications and wall stiffening. It has been suggested that polyamine oxidase (PAO) is important in producing H_2O_2 in vivo during cell growth and differentiation and host-pathogen interaction (Cervelli et al. 2001). Polyamines were considered as antioxidant molecule which inhibit lipid peroxidation in rat liver microsomes and in phospholipid vesicles. Polyamines have also been reported as direct free radical scavengers or to function as scavengers by interacting with other molecules (Benavides et al. 2000). In general, polyamine metabolism has long been known to distort in plant cells responding to insightful changes in plants interacting with fungal and viral pathogens (Walters 2003). To the best of our knowledge there is no report on lipoxygenase and polyamine metabolism in castor during wilt pathogen interaction. Therefore, present investigation was performed to study the role of lipoxygenase and polyamine in castor for wilt disease resistance.

Materials and methods

Castor genotypes and its inoculation with *Fusarium oxysporium* f. sp. *ricini*

The investigation was carried out using the four castor genotypes. The seeds of two resistance genotypes (SKP-84, 48–1) and two susceptible genotypes (VP-1, VI-9) to castor wilt were procured from the Main Castor and Mustard Research Station, Sardar Krushinagar Dantiwada Agricultural University, Sardar Krushinagar, Dantiwada, Gujarat, India.

Seeds were sown on the surface of the fine sterilized sand and then covered with a 2 cm layer of sand. The crop was well irrigated regularly to avoid any physiological stress and to maintain high relative humidity condition. Highly susceptible genotypes VP-1, VI-9 and resistant genotypes SKP-84, 48–1 were sown in individual trays.

All the four genotypes were infected with *Fusarium oxysporium* f. sp. *ricini* by mechanical method at 20 days after germination. Plants were pulled out gently from the sand and roots were washed with distilled water and roots from terminal sites about 1–2 cm were slotted out and injured roots of castor seedlings were dipped in 1×10^{-6} fungal suspension for 10–15 min, while for control treatment the injured roots were dipped in distilled water. After that plants were transplanted in fresh sterilized pot containing sterilized soil and sand in 1:1 ratio. For the all biochemical estimations i.e. lipoxygenase, polyamine oxidase activity, lipid peroxidation product and polyamines, second fresh leaves were taken at 0, 5 and 10 days after infection (DAI) while roots were taken at only at 0 and 10 DAI to remain the same plant for all the stages (0, 5 and 10 DAI) of analysis. All these parameters were estimated from both infected and non infected plants.

Lipoxygenase (LOX) activity: (EC 1.13.11.12)

Enzyme extraction

The leaves (0.2 g) were homogenized with 2 ml of 0.2 M sodium phosphate buffer (pH 6.5) using a pre-chilled mortar and pestle. The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C. The supernatant was used as the enzyme source (Mahatma et al. 2011).

Preparation of substrate

Stock solutions of 10 mM 3-Methyl-2-benzothiazolinone hydrazone (MBTH) and 5 mg/ml hemoglobin (100X their final concentrations) were made by dissolving the reagents in water. A solution containing 20 mM 4-(Dimethylamino) benzoic acid (DMAB) was prepared by dissolving 330 mg DMAB in 5 ml of 1 N HCl. This was then diluted to about 80 ml with water and 1.42 g of Na_2HPO_4 (100 mM) was added. The pH was adjusted to 6.0 with HCl and the volume brought to 100 ml with water. A 25 mM stock solution was prepared by adding 155 μ l (140 mg) of linoleic acid and 257 μ l (280 mg) of tween-20 to 5 ml of water. The mixture was emulsified by drawing back and forth in a Pasteur pipette and clarified by adding 0.6 ml of 1 N NaOH then after diluted to the final volume of 20 ml.

Assay

Solution A was prepared by mixing 10 ml of the 20 mM DMAB, 100 mM phosphate buffer solution (pH 6), 0.4 ml of the 25 mM linoleic acid stock, and 9.6 mL of water. Solution B was prepared by mixing 0.4 ml of 10 mM MBTH, 0.4 ml of 5 mg/ml hemoglobin, and 19.2 ml water. For the standard two-step assay, the sample, in a volume of 80 to 100 μ l was incubated with 4.0 ml of solution A. After incubation for the 7 min, 4.0 ml of solution B was added. After an additional 7 min, 1.9 ml of 1 % (w/v) sodium lauryl sulfate was added to terminate the reaction and absorbance was recorded at 598 nm (Anthon and Barrett 2001).

Polyamine oxidase (PAO) activity: (EC 1.5.3.3)

Enzyme extraction

The leaves (0.1 g) were homogenized with 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) using a pre-chilled mortar and pestle. The homogenate was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatant was used as the enzyme source (Kaur-Sawhney et al. 1981).

Enzyme assay

PAO activity was determined by peroxidase/guaiacol method. The rate of peroxidative oxidation of guaiacol by H_2O_2 released in enzyme extracts from leaf homogenates was assessed with spermidine as substrate. The reaction mixture consisted of 1 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.05 ml each of 25 mM guaiacol and 1 mg/ml of peroxidase (Horseradish Type II, Sigma) and 0.1 ml of crude enzyme. After pre incubation of mixture at room temperature for 2 min, 0.02 ml of 10 mM spermidine was added and absorbance was measured at 470 nm (Smith 1972).

Determination of thiobarbituric acid reactive substances (TBARS) / malondialdehyde (MDA)

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) described by Heath and Packer (1968).

Polyamines profiling through HPTLC

Extraction of polyamines

Plant tissues were extracted in 5 % cold (perchloric acid) $HClO_4$ at a ratio of about 100 mg/ml $HClO_4$ and place the extract in an ice bath for 1 h. Samples were pelleted at

48,000 g×20 min, and the supernatant phase containing the free polyamines fraction was stored at $-20\text{ }^{\circ}\text{C}$ in plastic vials.

Sample preparation

The method of Seiler and Wiechmann (1967) was adapted for plant tissues with some modifications. The quantity of 0.2 ml of $HClO_4$ extract were mixed with 400 μl of dansyl chloride (5 mg/ml in acetone, prepared fresh), and 200 μl of saturated sodium carbonate. After brief vortexing, the mixture was incubated in darkness at room temperature for overnight. Excess dansyl reagent was removed by reaction with 100 μl (100 mg/ml) of added proline, and incubated for 30 min. Dansyl polyamines were extracted in 0.5 ml benzene and vortexes for 30 s. The organic phase was collected and stored in glass vials at $-20\text{ }^{\circ}\text{C}$. Dansylated extracts were stable for up to 1 month. Standards were processed in the same way, and 20 nMol were dansylated for each, alone or in combinations. TLC was performed on high resolution silica gel 60 F 254.

HPTLC profiling of polyamines

Sample application

Extracted samples (15.0 μl) were sprayed as 1×9 mm bands along with standards (15.0 μl) on TLC Silica gel 60 F 254

Fig. 1 a: Lox activity in Leaf at 0, 5 and 10 days after infection. **b:** Lox activity in Root at 0, and 10 days after infection. NB: VP-1 and VI-9 Wilt Susceptible genotypes, 48-1 and SKP-84 – Wilt resistant genotypes. NI= Non- infected, I=Infected. The values are significantly different according F-test (0.05) and C.D. values are depicted as bar on column

plates 20×10 cm (silica gel plates, Merck) by CAMAG Linomat 5 applicator using nitrogen gas. The plates were activated at 100 °C for 10 min before sample application.

Separation of polyamines

Solvent system was used as described by Seiler (1971). Polyamines were separated by the solvent system chloroform: triethylamine (25:2, v/v). Solvent mixture was filled in the CAMAG Twin trough chamber (20×10 cm) and left overnight for saturation. The plates were placed in tank to separate polyamines until solvent reached 8 cm from origin.

Qualitative and quantitative analysis

After separation of polyamines, HPTLC plates were photographed using CAMAG Reprostar 3 system at 366 nm in fluorescent light. Quantitative determination was carried out using CAMAG TLC Scanner 3 at 350 nm.

Statistical analysis

All the biochemical parameters, except polymine profiling were analysed in three repetitions from two consecutive

experiments. Cumulative data of three repetitions from two experiments were taken as mean. The data obtained by MDA constituents and enzymes determination were subjected to simple completely randomised design (CRD) for the significance of various data using “F” test and compared by C.D value at 5 % level (Gomez and Gomez 1984).

Results and discussion

Lipoxygenase (LOX) activity

The lipoxygenase activity was higher in the leaves of resistant genotypes (18.32–19.47 Units min⁻¹ g⁻¹ protein) compared to susceptible genotypes (15.46–15.58 Units min⁻¹ g⁻¹ protein) at 0 DAI (Fig. 1a) and similarly higher activity of LOX was observed in the roots of resistant genotypes (Fig. 1b). Leaves of infected resistant genotypes showed appreciable higher activity of LOX (246.14–284.73 Units min⁻¹ g⁻¹ protein) at 5 DAI compared to infected susceptible genotypes (179–185 Units min⁻¹ g⁻¹ protein). Although, LOX activity was also increased in non- infected leaves after 5 DAI compared to 0 DAI but it was 0.5–0.7 fold lower than infected resistant genotypes. This increased

Fig. 2 a: PAO activity in leaf at 0, 5 and 10 days after infection. **b:** PAO activity in Root at 0 and 10 days after infection. NB: VP-1 and VI-9 Wilt Susceptible genotypes, 48-1 and SKP-84 – Wilt resistant

genotypes. NI=Non- infected, I=Infected. The values are significantly different according F-test (0.05) and C.D. values are depicted as bar on column

activity in non-infected leaves might be attributed due to wounding treatment of roots at 0 DAI which also causes stress on plants, which is in agreement with other studies which showed that wounding stress can induce PAL expression and activity (Huang et al. 2010). The LOX activity was significantly reduced in the leaves and roots of resistant and susceptible genotypes at 10 DAI. However, infected leaves and roots of resistant genotypes possessed significantly higher LOX activities than susceptible genotypes and their non-infected control at 10 DAI. Infected leaves of resistant genotypes had 28–35 % higher LOX activity, while infected roots of resistant genotypes showed about 2 fold higher LOX activity than susceptible genotypes at 10 DAI.

Increased LOX activity in plant pathogen interaction is well established (Keppler and Novacky 1986; Jain et al. 2002; Babita et al. 2006; Mahatma et al. 2011). A higher level of LOX activity was observed in *Fusarium udum* inoculated resistant pigeon pea cultivar than in susceptible cultivar. LOX activity increased progressively starting from 1st day of infection, reached maximum by 4th day and declined gradually thereafter. (Devi et al. 2000).

The increase in lipoxygenase activity suggests its possible involvement in imparting resistance following *Fusarium*

oxysporum f. sp. *ricini* infection. The increased LOX may generate signal molecules such as jasmonic acid, methyl jasmonic acid /or lipid peroxides, which co-ordinately amplify specific responses. Alternatively, LOX catalyzed reactions can result in the production of toxic volatile and non-volatile fatty acid derived secondary metabolites that can directly attack invading pathogens (Croft et al. 1993). Increased LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents (MacCarrone et al. 2000).

Polyamine oxidase (PAO) activity

At pre-infection, PAO activity was higher in the leaves of resistant genotypes (10.30–10.48 Units min⁻¹ g⁻¹ protein) compared to susceptible genotypes (6.42–6.94 Units min⁻¹ g⁻¹ protein) and similar trend was observed in the roots of resistant genotypes (22.68–26.44 Units min⁻¹ g⁻¹ protein) compared to susceptible genotypes (18.01–19.77 Units min⁻¹ g⁻¹ protein). PAO activity was drastically increased in the infected leaves of both resistant and susceptible genotypes as compared to their non-infected leaves at 5 DAI (Fig. 2a). But at this stage, constitutive levels of PAO in

Fig. 3 a: MDA constituent in Leaf at 0, 5 and 10 days after infection. **b:** MDA constituent in Root at 0 and 10 days after infection. NB: VP-1 and VI-9 Wilt Susceptible genotypes, 48-1 and SKP-84 – Wilt resistant

genotypes. NI=Non-infected, I=Infected. The values are significantly different according F-test (0.05) and C.D. values are depicted as bar on column

resistant genotypes were about 24–69 % higher than the susceptible genotypes. PAO activity was also higher in infected roots of all genotypes than non-infected roots at 10 DAI (Fig. 2b). However, roots of resistant genotypes had significantly higher activity than susceptible genotypes. Similarly, increased activities of the catabolic enzymes DAO and PAO were observed during HR induction in powdery mildew resistant varieties of barley at 1–3 days after inoculation (Cowley and Walters 2002b).

Although, PAO activity significantly declined in resistant and susceptible genotypes at 10 DAI but still it was higher

in infected leaves as compared to non-infected leaves of 5 DAI. In the present study, higher activity of PAO in resistant genotypes reflects lignifications of tissues, and is spatially and temporally associated with cell wall strengthening reactions involving cell wall peroxidases (Rodriguez-Kessler et al. 2008). Resistant genotypes showed the first line of defense against the pathogenic challenge which is associated with the plant cell wall, in which certain modifications take place during infection, respecting the rule “the harder the better.” Similarly, Yoda et al. (2003 and 2006) observed increased *PAO* expression and PA titers in *Nicotiana*

Fig. 4 Polyamine profiling of wilt resistant and susceptible genotypes of castor using HPTLC. Polyamines were detected at 350 nm in fluorescent wavelength

Table 1 Polyamines profiling of castor genotypes at pre-infection (I 0 h) and post-infection (II 5 days) and post-infection (III 10 days) stage (Individual polyamines and % area was detected by CAMAG SCANNER 3 at 350 nm)

Sr. No.	Genotypes	Stage	% Area of known polyamines				
			Polyamines	Leaf non infected	Root non infected	Leaf infected	Root infected
1	VP-1	I 0 Day	Cad	—	—	—	—
			Put	3.56	—	—	—
			Spd	7.44	19.77	—	—
		II 5 Day	Spm	—	—	—	—
			Cad	—	—	—	—
			Put	5.95	—	8.96	—
		III 10 Day	Spd	16.82	—	23.80	—
			Spm	5.77	—	10.56	—
			Cad	—	—	—	—
			Put	—	3.52	—	—
			Spd	9.59	10.81	6.62	3.42
			Spm	7.45	—	6.65	—
2	VI-9	I 0 Day	Cad	—	—	—	—
			Put	9.24	3.07	—	—
			Spd	23.92	5.21	—	—
		II 5 Day	Spm	12.22	—	—	—
			Cad	—	—	—	—
			Put	19.38	—	8.59	—
		III 10 Day	Spd	10.46	—	18.70	—
			Spm	10.25	—	17.51	—
			Cad	—	—	—	—
			Put	—	2.21	—	—
			Spd	17.99	9.58	7.75	—
			Spm	13.54	—	8.38	11.55
3	48-1	I 0 Day	Cad	—	—	—	—
			Put	8.83	19.31	—	—
			Spd	22.34	18.10	—	—
		II 5 Day	Spm	12.78	6.81	—	—
			Cad	—	—	—	—
			Put	10.44	—	6.44	—
		III 10 Day	Spd	30.51	—	21.55	—
			Spm	15.75	—	12.40	—
			Cad	—	—	—	—
			Put	3.25	2.86	2.89	2.66
			Spd	13.07	15.71	17.00	18.76
			Spm	13.83	—	16.55	—
4	SKP-84	I 0 Day	Cad	—	—	—	—
			Put	4.97	—	—	—
			Spd	16.52	6.25	—	—
		II 5 Day	Spm	7.75	2.75	—	—
			Cad	—	—	—	—
			Put	9.05	—	7.65	—
		III 10 Day	Spd	15.12	—	2.13	—
			Spm	12.30	—	3.56	—
			Cad	—	—	—	—
			Put	3.70	6.11	4.10	5.63
			Spd	10.43	14.09	25.71	17.15
			Spm	15.66	11.34	20.88	—

tabacum plants resistant to TMV. Cell death caused by TMV infection or cryptogein, an oomycete-originated elicitor, was partially mediated by H_2O_2 generated through PA catabolism. Interestingly, Kusano et al. (2008) suggested that H_2O_2 produced by PAO could trigger activation of Mitogen Activated Protein Kinases (MAPKs). *PAO* genes in *Arabidopsis* are upregulated by elicitors, such as flagellin, thus promoting probably activation of MAPKs involved in pathogen signal perception. Plants employ polyamine catabolism-derived H_2O_2 as a defensive tool upon exposure to biotic stresses has been reviewed by other groups (Walters 2003; Cona et al. 2006)

Malondialdehyde (MDA) content

Membrane lipid peroxidation was estimated as the content of MDA to observe membrane damage. The MDA content was higher in susceptible genotypes (367–375 $\mu\text{mol g}^{-1}$ FW in leaves and 148–152 $\mu\text{mol g}^{-1}$ FW in roots) compared to resistant genotypes (308–311 $\mu\text{mol g}^{-1}$ FW in leaves and 107–128 $\mu\text{mol g}^{-1}$ FW in roots) at pre-infection (20 DAS) stage. While, after infection (5 and 10 DAI) the MDA content was increased in infected leaves of resistant and susceptible genotypes as compared to their non-infected leaves (Fig. 3a). Moreover, leaves of infected resistant genotypes had about 1.5–1.7 times more MDA content than infected susceptible genotypes and non-infected resistant genotypes. The MDA content was declined significantly in infected and non-infected tissues of both resistant and susceptible genotypes at 10 DAI compared to 5 DAI. However, the MDA content was higher in both leaves and roots tissues (Fig. 3b) of resistant genotypes at 10 DAI as compared to (0 DAI) pre infection. The rate of lipid peroxidation product increased with *Fusarium oxysporum* f. sp. *ricini* infection in incompatible interaction whereas the rate of lipid peroxidation product decreased in compatible interaction. Higher activity of lipoxygenase was observed with increased level of MDA in tomato leaves as the results of *Fusarium oxysporum* infection (EI-Khallal 2007). In the present study, higher lipid peroxidation is accompanied by higher LOX activity in resistant genotypes of castor proved to be induced by pathogen and the subsequent products have been shown to possess antibacterial properties (Croft et al. 1993) and signaling functions (Melan et al. 1993)

Polyamines profiling through HPTLC

Results of polyamines profiling showed that the cadaverine was not detected in any castor genotype at any stage of analysis (Fig. 4). While, primary diamine putrescine was not detected in non-infected leaf tissues of susceptible genotypes at 10 DAI (Table 1) but it was present in root tissues

of same genotypes. Putrescine was only detected in infected roots of resistant genotypes of castor with low pace at 10 DAI. In the infected leaf tissues of susceptible genotypes putrescine, spermidine and spermine was increased at 5 DAI while these polyamines were decreased in resistant genotypes compared to their non-infected leaves. Interestingly, the levels of spermidine and spermine were increased in infected leaves of resistant genotypes at 10 DAI whereas reverse trend was observed in susceptible genotypes. The resistant genotypes had 2–3 times higher content of spermidine and spermine. Similar results were observed in an incompatible interaction between barley and powdery mildew, levels of free spermidine and of conjugated forms of putrescine and spermidine were found to increase after inoculation (Cowley and Walters 2002a). Furthermore, these results are in agreement with a previous report where the over expression of a key polyamine biosynthetic gene arginine decarboxylase (*adc*) in transgenic eggplant exhibited increased resistance against *Fusarium* wilt as compared to wild type seedlings. Similarly, the introduction of *samdc* gene in tobacco conferred resistance against *Fusarium* and *Verticillium* wilts (Wai and Rajam 2003). Higher content of spermine plays a role as a mediator in defence signalling against plant pathogens (Yamakawa et al. 1998; Takahashi et al. 2003). The levels of PAs, particularly PA conjugates are known to increase during fungal infections and are implicated in providing resistance against various plant pathogens. Therefore, high titers of PAs, in the incompatible interaction suggest their involvement in fungal resistance. The ‘spermine signaling pathway’ involves accumulation of spermine in the apoplast, upregulation of a subset of defence-related genes such as those encoding pathogenesis-related (PR) proteins, PR-1, PR-2, PR-3 and PR-5 (Yamakawa et al. 1998) and mitogen-activated protein kinases, and a type of programmed cell death known as the hypersensitive response. This response is triggered by spermine-derived H_2O_2 , produced through the action of polyamine oxidase (PAO) localized in the apoplast (Kusano et al. 2008; Moschou et al. 2008). Taken together, these data indicate double-edged roles of spermine in cell survival: as a free radical scavenger in the nucleus and as a source of free radicals in the apoplast, the interaction of spermine with other molecules is involved in the cell death.

The observed results of PAs are correlated with the polyamine oxidase (PAO) activity in susceptible and resistant genotypes in present study. This might be due to higher PAO activity of resistant genotypes utilized the higher content of polyamines as substrate at 5 DAI. The levels of polyamines in infected resistant genotypes at 10 DAI were almost similar to the level of polyamines in the susceptible genotypes at 5 DAI. Thus, resistant genotypes maintained the PAs and PAO homeostasis in both infected and non-infected tissues.

Acknowledgement Authors are thankful to Dr. P.S. Patel, Research Scientist, Main Castor Mustard research Station, Sardar Krushinagar Dantiwada Agricultural University, S.K. Nagar, Gujarat, India for providing genotypes of Castor.

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