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Original scientific paper

Antifungal activity of chitosan against *Alternaria alternata* and *Colletotrichum gloeosporioides*

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SUMMARY

Chitosan and its derivatives have been reported as a promising alternative for control of postharvest fungal pathogens. The objective of this study was to evaluate *in vitro* and *in situ* antifungal activity of chitosan against *Alternaria alternata* and *Colletotrichum gloeosporioides* isolated from decayed apple fruits. The fungi were tested *in vitro* using PDA medium with three concentrations of chitosan (1, 2 and 3 mg/ml). Fungal growth of the test pathogens was significantly affected by all chitosan doses ($P<0.05$) after 7 days of incubation at 25°C. Water solution of 3 mg/ml of chitosan inhibited completely the conidial germination of *A. alternata* and *C. gloeosporioides* after 18 h incubation at 25°C. The results obtained from biocontrol assay indicate that the inhibition of postharvest decay of *A. alternata* and *C. gloeosporioides* was significantly influenced by chitosan concentrations. Disease incidence in chitosan-treated fruit after 7 days incubation at 25°C was significantly lower than in the positive control for both fungi tested ($P<0.05$). *A. alternata* and *C. gloeosporioides* used in this study were progressively inhibited *in vitro* and *in situ* with increasing concentrations of chitosan from 1 to 3 mg/ml.

Keywords: Chitosan; Antifungal activity; Postharvest pathogens; Apple fruits

INTRODUCTION

Synthetic fungicides have been traditionally used to control and suppress postharvest diseases (Eckert & Ogawa, 1988). However, the development of fungicide resistance by postharvest pathogens and an increasing environmental concern over fungicide residues in food have prompted a search for alternative means for controlling postharvest decay (Holmes & Eckert, 1999). Over the past few decades, many attempts have been made to develop non-fungicidal methods to control postharvest decay on various commodities. They include environmental modifications, such as storing commodities at temperatures suppressive to pathogen development, modifying relative humidity or the atmosphere, treatments with hot air or water, inducing resistance by applying elicitors or UV irradiation, and applying substances generally regarded as safe (GRAS), (Janisiewicz & Korsten, 2002).

Chitosan is a linear polysaccharide consisting of β-(1-4)-linked 2-amino-2-deoxy-D-glucose residues, originating from deacetylated derivative of chitin, which is the second most abundant polysaccharide in nature after cellulose (Jianglian & Shaoying, 2013). It is non-toxic, biodegradable, biofunctional and biocompatible. Chitosan is produced from chitin, which is usually obtained from shells of krill, shrimp, crab, insect cuticle and mushroom cell wall (Rinaudo, 2006). The International Commission on Natural Health Products

(1995) recognized chitin as a natural product for the 21st century, and in 2005 chitosan was approved as GRAS by the US Food and Drug Administration based on scientific procedures for use in foods (Munoz et al., 2009).

Regarding the primary structure of chitosan, its polycationic nature allows this polymer to perform numerous and unique biological functions with great potential applicability in various industries including agriculture, medicine and pharmacology (Wojdyła, 2004; Badawy et al., 2005; Rinaudo, 2006; Tayel et al., 2010; Choi et al., 2016).

In particular, the antifungal effects of chitosan and its derivatives have attracted considerable attention over the past several decades. This natural compound has proved to be effective in preventing fungal growth by directly interfering in or by activating certain biological processes (El Ghaouth et al., 1992). Five main modes of action of chitosan include: electrostatic interactions, plasma membrane damage mechanism, chitosan-DNA/RNA interactions, metal chelation capacity of chitosan, and deposition on microbial surface (Xing et al., 2015). Chitosan is known to elicit many plant defense responses by activating pathogenesis-related (PR) gene functions, such as chitinases (Benhamou & Theriault, 1992), β-glucanases, lignin (Notsu et al., 1994) and callose (Kauss et al., 1989).

Chitosan has strong antimicrobial activity that could effectively control postharvest fruit decay (Aider, 2010). This compound also maintains the quality of fruits and extends their shelf life (Kumar et al., 2017).

To our knowledge, there are no publications on the control of postharvest decay of fruits in Serbia using chitosan. Therefore, the objective of this study was to investigate *in vitro* and *in situ* the antifungal activity of chitosan against *Alternaria alternata* and *Colletotrichum gloeosporioides* originating from apple fruits.

MATERIAL AND METHODS

Chitosan

Medium molecular weight chitosan (190.000-310.000 Da; degree of deacetylation 75-85%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Stock solution of chitosan (10 mg/ml) was made by dissolving 1 g of chitosan powder in 100 ml of purified water containing 0.5 ml (v/v) glacial acetic acid (CH_3COOH). The solution was stirred for 5 h on a magnetic stirrer (iStir MS10, Neuation Technologies Pvt. Ltd, India). Solution pH was adjusted to 5.6 by adding 1N NaOH, and using a digital pH meter (inoLab ph Level 1, WTW GmbH & Co. KG, Germany). The solution was autoclaved for 15 min at 121°C. After autoclaving, aliquots of chitosan stock solution were taken and mixed with sterilized PDA medium and sterilized distilled water to obtain different chitosan concentrations (1, 2 and 3 mg/ml).

Pathogens

A. alternata and *C. gloeosporioides* were isolated from decayed apple fruits. Stock cultures of pathogens were maintained on potato dextrose agar (PDA) at 4°C, and kept in the Culture Collection of the Institute for Plant Protection and Environment. Working cultures were prepared by transferring stock agar plugs containing mycelia of each pathogen onto PDA and incubating them for 7 days at 25°C in the dark.

Conidial suspension of *A. alternata* and *C. gloeosporioides* were prepared as follows: the pathogens were grown on PDA under constant fluorescent light. After 2-weeks incubation at 25°C, spores were harvested by flooding the plates with 5 ml of sterile distilled water containing 0.1% (v/v) Tween 80, scraping with a rubber spatula, and then filtering the suspension through double layers of cheesecloth. The spores were counted with a haemocytometer, and adjusted with sterile distilled water to $1x10^6$ conidia/ml.

Effect of chitosan on A. alternata *and* C. gloeosporioides *in vitro*

The effect of chitosan on mycelial growth of the test pathogens was estimated in a radial growth inhibition assay. Five mm agar discs from actively growing PDA cultures of *A. alternata* and *C. gloeosporioides* were transferred to the surface of PDA plates containing different concentrations of chitosan (1, 2 and 3 mg/ml). PDA plates without chitosan inoculated with test pathogens served as controls. All Petri plates were incubated at 25°C for 7 days in the dark. Mycelial growth was determined by measuring colony diameters.

Micro-morphology of *A. alternata* and *C. gloeosporioides* was observed 14 days after incubation on PDA with all tested concentrations of chitosan. Fungal mycelia were deposited on the surface of microscopic slides containing lactophenol cotton blue. Changes in hyphae and conidia morphology were observed using an Olympus BX51 microscope (Olympus Corporation, Japan).

To assess the effects of chitosan on spore germination, 50 µl of conidial suspension of *A. alternata* and *C. gloeosporioides* ($1x10^6$ conidia/ml), and 50 µl of water solution of chitosan at different concentrations (1, 2 and 3 mg/ml) were mixed and transferred to sterile

microscope slides. The control consisted of suspensions of pathogen conidia in sterile distilled water. The slides were placed inside Petri plates and incubated under high humidity at 25°C. After 18 h incubation, 100 spores of each fungal pathogen were measured for germination rate. Spores were considered germinated when germ tube length was equal to or greater than spore length.

Biocontrol assay

For biocontrol assay, apple fruits (cv. 'Golden Delicious') were washed in running water, dipped in ethanol (70%) for 2 min, rinsed twice with distilled sterile water and air-dried. After drying, apple fruits were wounded (5 mm deep and 5 mm wide) with a sterile cork borer at equator region. Aliquots of 50 µl of water solution of chitosan at different concentrations (1, 2 and 3 mg/ml) were pipetted into each wound. After 1 h, 50 µl of conidial suspension of *A. alternata* or *C. gloeosporioides* (1x10^6 conidia/ml) were pipetted into each wound. Positive control fruits were inoculated with fungal conidial suspension only, and negative control with sterile distilled water. The fruits were placed on moist filter paper in plastic containers and incubated at 25°C. After 7 days the diameters of necrotic lesions were measured.

Statistical analysis

All experiments were repeated twice with three replications of each treatment. Data were analyzed by one-way analysis of variance (ANOVA). Mean values were compared using Tukey's test and significance was evaluated at $P<0.05$. Statistical analysis was performed using the statistical software Minitab 18 (Minitab, Inc, USA).

RESULTS

Effect of chitosan on A. alternata *and* C. gloeosporioides *in vitro*

The antifungal activity obtained by radial growth inhibition assay on PDA with different concentrations of chitosan is reported in Table 1. The results indicate that *A. alternata* and *C. gloeosporioides* used in this study were progressively inhibited with increasing concentrations of chitosan from 1 to 3 mg/ml. Fungal growth of the tested pathogens was significantly affected by all chitosan doses ($P<0.05$) after 7 days of incubation (Figure 1). Chitosan showed better inhibitory effect on *A. alternata*.

Table 1. Effects of chitosan on *A. alternata* and *C. gloeosporioides* growth *in vitro*

Treatment	*A. alternata* growth (mm)	*C. gloeosporioides* growth (mm)
Control	76.33 ± 0.58 a	90.00 ± 0.00 a
Chitosan 1 mg/ml	38.50 ± 0.87 b	80.67 ± 1.16 b
Chitosan 2 mg/ml	19.50 ± 0.50 c	49.00 ± 1.00 c
Chitosan 3 mg/ml	9.00 ± 0.00 d	20.33 ± 0.58 d

Data represent standard deviations of the means
Means in columns marked by different letters are significantly different according to Tukey's multiple range test ($P\leq0.05$)

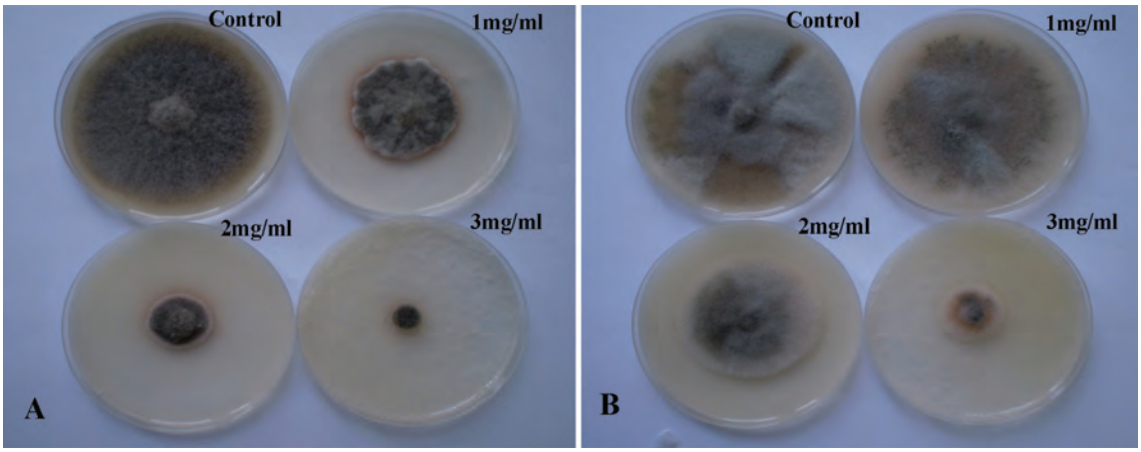

Figure 1. Effects of chitosan solution (1, 2 and 3mg/ml) on mycelial growth of *A. alternata* (A) and *C. gloeosporioides* (B) *in vitro*

Table 2. Effects of chitosan on spore germination of *A. alternata* and *C. gloeosporioides*

Treatment	*A. alternata* spore germination (%)	*C. gloeosporioides* spore germination (%)
Control	92.33 ± 2.52 a	97.33 ± a
Chitosan 1 mg/ml	40.67 ± 3.06 b	52.33 ± b
Chitosan 2 mg/ml	18.67 ± 1.16 c	22.67 ± c
Chitosan 3 mg/ml	0.00 ± 0.00 d	0.00 ± d

Data represent standard deviations of the means
Means in columns marked by different letters are significantly different according to Tukey's multiple range test (P≤0.05)

Table 3. Effects of chitosan on *A. alternata* and *C. gloeosporioides* decay on apple fruits

Treatment	*A. alternata* lesion diameter (mm)	*C. gloeosporioides* lesion diameter (mm)
Control +	26.33 ± 0.57 a	33.50 ± 1.32 a
Chitosan 1 mg/ml	21.50 ± 0.50 b	26.00 ± 1.00 b
Chitosan 2 mg/ml	17.50 ± 1.32 c	17.67 ± 1.55 c
Chitosan 3 mg/ml	8.33 ± 1.15 d	11.00 ± 1.00 d
Control -	0.00 ± 0.00 e	0.00 ± 0.00 e

Data represent standard deviations of the means
Means in columns marked by different letters are significantly different according to Tukey's multiple range test (P≤0.05)

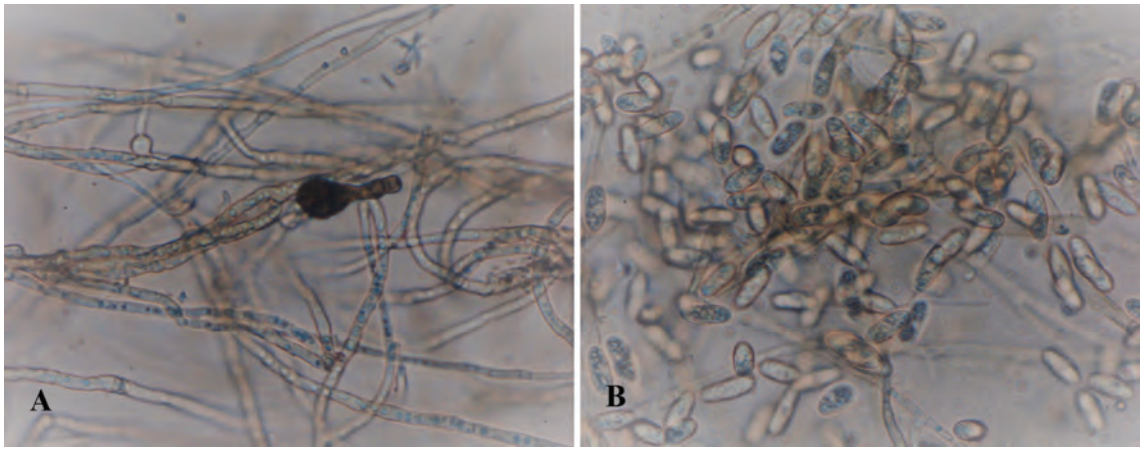

Figure 2. Micro-morphology: (A) malformation of *A. alternata* on PDA with chitosan (3mg/ml); (B) non-germinated conidia of *C. gloeosporioides* in water solution of chitosan (3 mg/ml), (1000 x magnification).

Microscopic examination showed that the presence of 3 mg/ml chitosan on PDA caused morphological changes, such as hyphal distortion, abnormal shapes and necrosis on both tested fungal pathogens (Figure 2A).

As shown in Table 2, spore germination of *A. alternata* and *C. gloeosporioides* were significantly inhibited by chitosan in a concentration-dependent mode ($P < 0.05$). Chitosan applied at the concentration of 3 mg/ml completely inhibited spore germination of the two tested fungi after 18 h incubation. The non-germinated conidia were deformed and changed shape (Figure 2B).

Biocontrol assay

In order to test the antifungal potential of chitosan *in situ*, we used a biocontrol assay on apple fruits. The results indicate that the inhibition of postharvest decay of *A. alternata* and *C. gloeosporioides* was significantly influenced by chitosan concentrations (Table 3). Disease incidence on chitosan-treated fruits was significantly lower than on the positive control for both fungi tested ($P<0.05$). Treatment with water solution of chitosan (3 mg/ml) efficiently protected

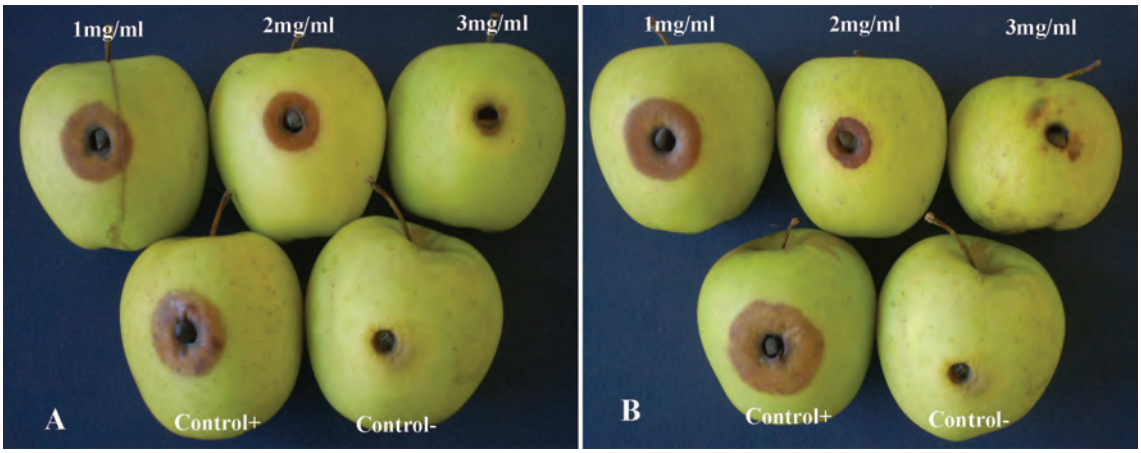

Figure 3. Effects of chitosan solution (1, 2 and 3mg/ml) on *A. alternata* (A) and *C. gloeosporioides* (B) decay on apple fruits *in situ*

apple fruits from decay and significantly inhibited necrosis, ranging from 8.33 ± 1.15 mm for *A. alternata* to 11.00 ± 1.00 mm for *C. gloeosporioides*. No lesions developed on negative control fruits inoculated with sterile distilled water.

Reductions in necrosis of apple fruit tissue obtained when chitosan (1, 2 and 3mg/ml) was used against *A. alternata* and *C. gloeosporioides* are shown in Figure 3. Importantly, a protecting effect of chitosan against the tested postharvest fungi was not only observed on the apple peel, but also deep inside the tissue.

DISCUSSION

In vitro, chitosan significantly inhibited fungal growth and spore germination of *A. alternata* and *C. gloeosporioides* at all tested concentrations compared to the control. The antifungal activity of chitosan and its ability to reduce *in vitro* growth of postharvest fungi has been demonstrated in other studies (Romanazzi et al., 2001). Most of the investigations revealed that chitosan affects mycelium growth, sporulation, morphology and molecular organization of fungi. Chitosan can effectively inhibit the development of phytopathogenic fungi at different life-cycle stages (Xing et al., 2015). For instance, chitosan completely inhibited spore germination, germ tube elongation, and mycelial growth of *Alternaria kikuchiana* and *Physalospora piricola* at 5 g/l *in vitro* (Meng et al. 2010). This compound also effectively inhibits radial mycelium growth, sporulation, and spore germination of *Alternaria* spp. (Bhaskara, et al, 1998), *Botrytis* spp. (Liu et al., 2007; Munhuweyi et al., 2016), *Rhizopus* spp. (Hernandez-Lauzardo et al., 2010), *Fusarium* spp., (Al-Hetar et al., 2011), *Penicillium* sp. and *Pilidiella granati* (Munhuweyi et al., 2016). Wang et al. (2013) reported an antifungal activity of chitosan against *P. expansum* isolated from jujube fruit. In the same study the authors found that chitosan disrupted plasma membrane of the fungal cell.

The results of our study showed that chitosan had a stronger inhibitory effect on spore germination than on mycelial growth of *A. alternata* and *C. gloeosporioides in vitro*. These results are not consistent with results reported by Meng et al. (2010), who suggested that chitosan and oligochitosan had stronger inhibitory effect on mycelia growth than on spore germination and germ tube elongation of both *A. kikuchiana* and *P. piricola*. Liu et al. (2007) reported that the sensitivity of *B. cinerea* and *P. expansum* to chitosan might vary with different development stages.

Microscopical examination in our study showed that the presence of chitosan on PDA medium caused hyphal distortion, abnormal shapes and necrosis of *A. alternata* and *C. gloeosporioides*. Effects of chitosan and its oligomers on fungal morphology are well documented (Hassan & Chang, 2017). Scanning electron microscope analyses revealed that inhibition of fungal growth in response to chitosan and its oligomers was accompanied with excessive mycelial branching, abnormal shapes, swelling and hyphal size reduction of *B. cinerea*, *R. stolonifer*, *A. alternata*, *P. expansum* (Oliveira Junior et al., 2012) and *P. italicum* (Lee et al., 2016). Chitosan layer around mycelial surface caused morphological anomalies, which make nutrient transport difficult. Chitosan is

also responsible for cytological changes, protoplasm dissolution and large fungal vesicles (Al-Hetar et al., 2011).

In the present study, treatments with chitosan reduced disease incidence caused by *A. alternata* and *C. gloeosporioides* in apple fruits. Fruits treated with the highest chitosan concentration (3mg/ml) showed a significant reduction in lesion diameter compared to control fruits. The protecting effect was observed not only on the apple peel, but also deep inside the tissue. Antifungal activity has been demonstrated *in situ* or *in vivo* in many different plant-pathogen systems. Treatments with chitosan reduced disease incidence and inhibited lesion expansion caused by these two fungal pathogens in pear fruit (Meng, et al., 2010). In commercial wine grapes, chitosan effectively inhibited the growth of *B. cinerea* in liquid culture and suppressed gray mold on detached grapevine leaves and bunch rot (Reglinski et al., 2010). This compound efficiently controlled postharvest gray (*B. cinerea*) and blue mold (*P. expansum*), and elicited defense response in kiwi fruit by inducing gene expression of certain enzymes (catalase, superoxide dismutase, and ascorbate peroxidase) (Zheng et al., 2017). Chitosan had a positive effect in terms of reduction in postharvest decay caused by *P. expansum* on jujube fruit (Wang et al., 2013). The authors also determined that chitosan had no negative impact on fruit quality. More than 60% control of anthracnose on papaya fruit was achieved when applied before C. *gloeosporioides* inoculation (Bautista-Banos et al., 2003). A significant reduction in anthracnose disease after coating tomato fruits and grape berries with 2.5% chitosan was reported by Munoz et al. (2009). Reductions in postharvest disease incidence and lesion diameters by chitosan application were also recorded in mango (Jitareerat et al., 2007).

Postharvest losses caused by fungal diseases are the major factor limiting the storage life of apple fruits (Manssouri et al., 2016). The results of the present study demonstrate that chitosan is effective in controlling *Alternaria* and *Colletotrichum* decay in apple fruit and could be considered as a potential natural agent to control postharvest diseases.

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Antifungalna aktivnost hitosana prema *Alternaria alternata* i *Colletotrichum gloeosporoides*

REZIME

Istraživanja pokazuju da su hitosan i njegovi derivati dobra alternativa u kontroli skladišnih fitopatogenih gljiva. Cilj ovog rada bio je da ispita antifungalnu aktivnost hitosana prema *Alternaria alternata* i *Colletotrichum gloeosporoides*, izolovanih sa inficiranih plodova jabuke, u *in vitro* i *in situ* uslovima. Rast gljiva je testiran *in vitro* koristeći PDA podlogu sa različitim koncentracijama hitosana (1, 2 i 3 mg/ml). Porast oba patogena je bio značajno smanjen ($P<0.05$) u svim koncentracijama hitosana nakon 7 dana inkubacije na 25°C. Vodeni rastvor hitosana koncentracije 3 mg/ml je u potpunosti inhibirao klijanje konidija *A. alternata* i *C. gloeosporoides* nakon inkubacije od 18 sati na temperaturi od 25°C. Rezultati ogleda *in situ* ukazuju da inhibicija propadanja plodova inficiranih sa *A. alternata* i *C. gloeosporoides* zavisi od koncentracije rastvora hitosana. Pojava bolesti na plodovima jabuka tretiranih rastvorom hitosana nakon 7 dana inkubacije na 25°C bila je značajno smanjena za obe vrste gljiva ($P<0.05$) u odnosu na pozitivnu kontrolu. Rast *A. alternata* i *C. gloeosporoides* je bio progresivno inhibiran *in vitro* i *in situ* sa povećanjem koncentracije rastvora hitosana, od 1 do 3 mg/ml.

Ključne reči: Hitosan; Antifungalno delovanje; Skladišni patogeni; Jabuka