DEHYDROGENAZE POLYMORPHISM AS A TOOL FOR EARLY SELECTION OF LOW VIGOUR ROOTSTOCK FOR SWEET AND SOUR CHERRY

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Abstract: The correlation of dehydrogenase isoenzyme profile with plant/tree vigour of twenty genotypes belonging to the genus *Prunus* and the subgenus *Cerasus* as well as to four commercial rootstocks (Colt, Gisela 5, Gisela 6 and MaxMa 14) was investigated in this study. Principal component analysis was applied for the classification of the cherry rootstocks based on vigour traits and polymorphism of isoenzymes in order to determine the most useful dehydrogenase enzyme in the preselecting process of cherry rootstocks. The most influential variables which led to the separation of low vigorous genotypes from other genotypes were isocitrate dehydrogenase, malate dehydrogenase and phosphogluconate dehydrogenase. Since both significant and negative correlations were observed between tree vigour and malate dehydrogenase, as well as between tree vigour and phosphogluconate dehydrogenase, those systems can be used for early selection of low vigour rootstocks. Our results suggest that the association between low vigour and malate dehydrogenase genotype *ab* provides a convenient marker that can be characterised even at the seedling stage, and could be applied in early rootstock selection and breeding programmes.

Key words: prediction, vigour, rootstock, isoenzyme, correlation, principal component analysis.

Introduction

Traditionally, the vigourous seedling rootstocks, Mazzard (*Prunus avium* L.) and Mahaleb (*Prunus mahaleb* L.), are commonly used for cherry budding and grafting. According to Lang (2000), these rootstocks have good compatibility with most cherry cultivars and good adaptability to different soil conditions. Their main

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disadvantages are high vigour, late entry into full bearing and unevenness of morphological and physiological characteristics of a grafted cultivar. According to Ercisli et al. (2006), growers in Turkey, which is the world leader in sweet cherry production, preferred to use Mazzard seedlings for sweet cherries because of its good soil adaptability, while *P. mahaleb* seedlings were used only on calcareous droughty soils. On the other hand, *P. mahaleb* still remains the main choice for sour cherry producers, because of its tolerance to drought, smaller tree size, good precocity and high productivity. Ercisli et al. (2006) have also stated that the use of low vigour rootstocks Gisela 5 and Gisela 6 is spreading very fast in Turkey.

Milošević et al. (2014) have reported that in Serbian sweet cherry orchards, the most widely used rootstocks are seedlings of *P. avium* L. (Mazzard), sporadically Colt, then seedlings of *P. mahaleb* L. (Mahaleb), Gisela 5 and MaxMa 14. Over the past 25 years, interest of Serbian sweet cherry growers in low and medium vigour rootstocks has increased due to the fact that smaller trees reduce production cost, especially training, pruning, harvesting as well as disease and pest protection.

In order to overcome the problem of vigorous trees, intensive work has been done on development of rootstocks with reduced vigour. In the last couple of decades, Hrotkó (2016) has analysed *P. fruticosa,* its hybrids collected from the natural flora of Hungary, and *P. fruticosa* × *P. mahaleb* hybrids in order to create dwarfing rootstocks. In Serbia, due to low vigour, 'Oblačinska' sour cherry (*P. cerasus*) has been traditionally used as a dwarfing rootstock for sweet and sour cherries. 'Oblačinska' is an autochthonous and heterogeneous cultivar, and is the most extensively planted sour cherry cultivar in Serbian commercial orchards. Trees have low vigour and are suitable for dense planting (Rakonjac et al., 2010).

The selection of dwarfing rootstocks for fruit crops has received great attention in the last decades. Reduced vegetative growth is a desirable trait that contributes to the higher productivity (even in the early years of orchard establishment), lower pesticide usage (Olmstead et al., 2004) and more cost-effective orchard maintenance (Seleznyova et al., 2008; Fassio et al., 2009). Dwarfing rootstocks also reduce labour costs by reducing canopy volume and lowering the fruiting zone to a height where a high percentage of fruit may be harvested (Lang, 2000; Whiting et al., 2005).

The prediction of the tree vigour potential is very important in rootstock breeding. Various prediction methods such as measurements of tree height, tree spread and trunk girth at the seedling stage (Miller, 1977), relationship between malate dehydrogenase (MDH) isozyme genotype and plant vigour (Werner and Moxley, 1991), anatomical characteristics of roots and stems (Zorić et al., 2011), hydraulic conductance of seedlings (Iwasaki et al., 2011) and root and rootstock stem hydraulic conductivities (Ljubojević et al., 2013) were applied in the previous studies.

Isoenzyme variability is an abundant source of genetic markers that can be used for early selection of genotypes with desirable economic traits. Since the relationship between isozyme variation and tree growth is well-known in peach, the correlation of isoenzyme profile with plant vigour of twenty *Prunus* (subgenus *Cerasus*) genotypes and four interspecies hybrids was investigated in this study. The main goal was to evaluate the potential application of isoenzymes in the preselecting process of low vigour rootstocks for cherries.

Materials and Methods

Plant material consisted of 20 cherry genotypes belonging to *Prunus cerasus* (8), *Prunus avium* (4), *Prunus fruticosa* (6), *Prunus mahaleb* (2), together with four interspecies hybrids (commercial rootstocks) including Colt, Gisela 5, Gisela 6 and MaxMa 14. *P. cerasus* ('Oblačinska' sour cherry), *P. avium*, *P. fruticosa* and *P. mahaleb* genotypes were selected from natural populations of Serbia. The selection was done according to the observed diversity in phenological and morphological traits of trees and fruits.

Tree vigour (TV), leaf length (LL) and leaf width (LW) were observed *in situ* for *Prunus sp*. genotypes and in a nursery for Colt, Gisela 5, Gisela 6 and MaxMa 14 rootstocks, during two consecutive years (2014 – 2015). A description of tree vigour (3 – weak; 5 – medium; 7 – strong; 9 – extremely strong) was based on descriptors provided by the International Union for the Protection of New Varieties of Plants (UPOV, 2006) and The International Board for Plant Genetic Resources (Schmid et al., 1985), while leaf length (LL) and leaf width (LW) were measured on a sample of 30 leaves per genotype, taken randomly from 1-year-old shoots. During the study, pruning and irrigation were not applied.

Seven dehydrogenase enzyme systems were analysed: alcohol dehydrogenase (ADH), glutamate dehydrogenase (GDH), isocitrate dehydrogenase (IDH), formate dehydrogenase (FDH), malate dehydrogenase (MDH), phosphogluconate dehydrogenase (PGD) and shikimate dehydrogenase (SDH). Two independent samples from each genotype were used for protein extraction. The inner bark from one-year-old shoots at the dormant stage was used for the extraction and evaluation of isoenzyme activity. Vertical polyacrylamide gel electrophoresis (PAGE) was used for the isoenzyme analysis. Polyacrylamide gel containing 8% acrylamide was used for separation. Sample preparation and staining procedures were done in accordance with the protocols given by Bošković et al. (1994) for stone fruit species. Electrophoresis was performed at +4 °C and consisted of three phases. Prior to sample loading, pre-electrophoresis was done for 45 minutes at 100 V. Afterwards, samples of 25 μl of enzyme extracts were loaded. The second phase lasted for 45 minutes at 100 V. The third stage was carried out at 400 V for 3 h. As for the majority of enzymes, staining was performed in accordance with the

protocol given by Bošković et al. (1994), and for FDH in accordance with Wendel and Weeden (1989). Gels were visually observed and bands that represented isoenzyme patterns were analysed. The loci of the same enzyme system were numbered progressively, from the slowest-migrating to the fastest-migrating form, beginning with locus 1. Data for dehydrogenase isoenzyme polymorphism and isoenzyme banding patterns of evaluated genotypes was taken from the previous study of Čolić et al. (2012). Numbers corresponding to isoenzyme banding patterns among each system represent different phenotypes, based on the number and position of bands on the gels.

For the purpose of statistical analysis, individual means for the traits and zymogram patterns were used. Relationships among the genotypes were investigated by principal component analysis (PCA). The correlation between the traits was determined using the Pearson correlation coefficient. Data processing was performed using the statistic programme 'Statistica' Version 6.0 for Windows (StatSoft, Inc., Tulsa, Oklahoma, USA).

Results and Discussion

Although the mechanism by which the rootstock regulates scion characteristics has not been identified, it is well known that rootstock influences the tree growth (Cantín et al., 2010), vigour, fruit and leaf properties (Rakonjac et al., 2016).

The morphological characteristics and isoenzyme profile of the studied genotypes are shown in Table 1. As expected, the wild cherry genotypes were characterised by the longest and widest leaves and strong tree vigour, ranging from medium to extremely strong. On the basis of tree vigour, sour cherry genotypes can be divided into two groups, having weak or medium vigour. Sour cherry leaves were smaller compared to sweet cherry, with LL ranging from 5.6 to 9.3 cm, and LW from 2.8 to 4.7 cm. Among studied genotypes, *P. mahaleb* was characterised by the most vigorous tree, while leaf measurements were similar to sour cherries. The lowest vigour, like in Gisela 5 and Gisela 6, was observed also in P. *fruticosa* genotypes, with exception of SV 3. Similarly, in this group, we observed the smallest leaves with the lowest values for LL and LW. Among studied rootstocks, the most vigorous was Colt, followed by MaxMa 14. Rootstocks Gisela 5 and Gisela 6 were distinguished by their lowest vigour.

Čolić et al. (2012) found significant dehydrogenase isoenzyme polymorphism in the genus *Prunus*, the subgenus *Cerasus*. In the studied material, seven dehydrogenase systems (ADH, GDH, IDH, MDH, PGD and SDH) were polymorphic, while FDH was monomorphic. The patterns of polymorphic systems for each of the studied genotypes are presented in Table 1.

IDH was found to be the most polymorphic dehydrogenase system represented with eight different phenotypes. Activity was visible in two loci: *Idh-1*, with two

alleles (*b* and *c*) and three phenotypes (*bb*, *bc* and *cc*). For locus *Idh-2*, we observed both genotypes with visible activity and genotypes without activity. Four phenotypes (*ab*, *ac*, *bb* and *bc*) were identified for this locus. Regarding all observed loci in this study, we found that *Idh-2* was the most polymorphic. Although great variability was established, we could not connect low tree vigour with any specific banding pattern.

Table 1. Morphological characteristics and isoenzyme banding patterns of the studied genotypes.

No.	Cultivar/ genotype	Species/ interspecific hybrid	Tree* vigour	Leaf length (cm)	Leaf width (cm)	Isoenzyme banding patterns**					
						ADH	GDH	IDH	MDH	PGD	SDH
1	DT X9	*P. avium*	5	8.8	5.5	5	1	5	1	1	7
2	DT X3	*P. avium*	7	11.0	5.8	5	1	5	1	1	7
3	DT X7	*P. avium*	7	9.9	5.2	5	2	5	1	1	7
4	DT K9	*P. avium*	9	10.4	6.1	5	4	11	1	1	7
5	Oblačinska UD 1	*P. cerasus*	5	8.9	3.9	1	1	8	2	2	2
6	Oblačinska UD 8	*P. cerasus*	5	8.2	4.2	1	1	10	2	2	1
7	Oblačinska UD 6	*P. cerasus*	3	8.2	4.3	1	1	7	4	2	2
8	Oblačinska D1 R	*P. cerasus*	5	8.1	4.2	1	1	10	2	2	2
9	Oblačinska D4 R	*P. cerasus*	5	5.9	2.8	1	1	10	2	2	2
10	Oblačinska II/10 R	*P. cerasus*	3	7.6	3.9	1	1	10	2	2	2
11	Oblačinska XI/3 R	*P. cerasus*	5	9.3	4.7	1	1	10	2	2	2
12	Oblačinska D4 RŠ	*P. cerasus*	3	8.1	4.2	1	1	10	2	2	2
13	BNS	*P. mahaleb*	9	6.1	3.8	2	6	2	1	3	13
14	Tika	*P. mahaleb*	7	5.2	4.7	6	1	10	3	3	3
15	SV 1	*P. fruticosa*	3	3.9	2.0	1	3	6	2	5	2
16	SV 2	*P. fruticosa*	3	4.8	2.5	1	3	6	2	5	2
17	SV 3	*P. fruticosa*	5	6.8	4.4	2	1	10	2	2	2
18	SV 4	*P. fruticosa*	3	4.2	2.1	2	1	10	4	5	2
19	SV 5	*P. fruticosa*	3	4.7	2.3	2	1	10	4	5	2
20	SV 7	*P. fruticosa*	3	5.9	2.8	2	1	4	2	4	2
21	Colt	*P. avium x P. seudocerasus*	7	9.3	5,0	2	2	2	1	2	4
22	Gisela 5	*P. cerasus x P. canescens*	3	6.1	3.3	1	3	10	4	2	1
23	Gisela 6	*P. cerasus x P. canescens*	3	6.5	3.8	1	1	7	4	2	1
24	Max Ma 14	*P. mahaleb x P. avium*	5	7.2	3.8	1	1	4	1	1	6

*Tree vigour (TV): 3 – weak; 5 – medium; 7 – strong; 9 – extremely strong; **Source: Čolić et al. (2012).

For MDH, the polymorphism and the presence of alleles *a* and *b* were observed in one region of activity, marked as *Mdh-1*. Homozygous allelic constitution *aa* was observed for vigorous *P. avium* and *P. mahaleb* (BNS) genotypes, and rootstocks Colt and MaxMa 14 where *P. avium* is one progenitor, while homozygous allelic constitution *bb* was observed only for one *P. mahaleb* genotype (Tika). Heterozygous allelic constitution *ab* characterised low vigour genotypes, *P. fruticosa,* 'Oblačinska' sour cherry clones and rootstocks Gisela 5 and Gisela 6, where *P. cerasus* was the female parent. Variability for *Mdh-1* was detected in 'Oblačinska' sour cherry and *P. fruticosa*, and it was expressed with two and/or four bands.

One polymorphic region *Pgd-1* of activity was observed on PGD zymograms. Three alleles, four phenotypes (*aa*, *ab*, *ac* and *bb*) and five banding patterns were distinguished. Homozygous allelic constitution *aa* (obtained for wild cherry) and *bb* (observed for *P. mahaleb*) corresponded with strong vigour. Contrarily, heterozygous allelic constitutions *ab* and *ac* corresponded with low vigour of 'Oblačinska' sour cherry, rootstocks Gisela 5, Gisela 6, and *P. fructicosa*.

PCA was performed to establish the relationship between the isoenzyme systems and tree vigour. The initial matrix of 24 (the number of samples) × 9 traits (TV, LL, LW, ADH, GDH, IDH, MDH, PGD and SDH) was processed. Since the first two principal components (PC1 and PC2) explained the greatest amount of variance (45.11% and 19.98%, respectively), the scatter plot of PC1/PC2 is presented as Figure 1. Starting from the negative to positive values of PC1, a trend of a general decrease of the tree vigour and leaf size can be noticed.
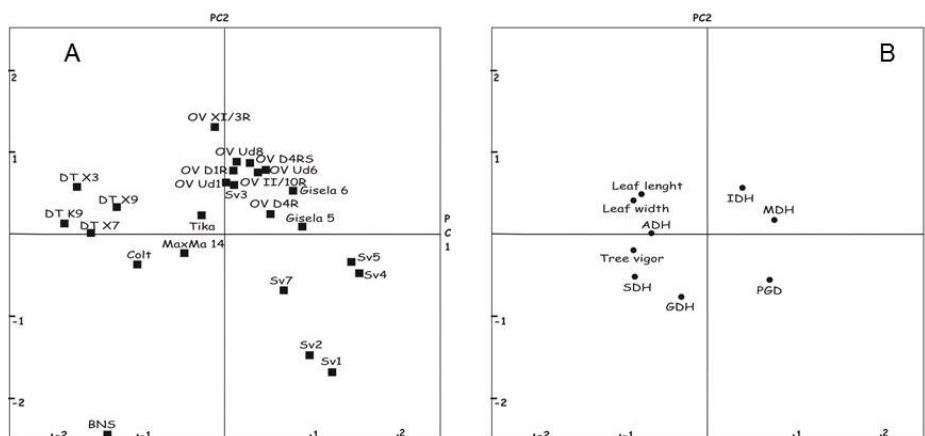

Figure 1. Principal component analysis, scores plot of the first two principal components (A) showing the clustering of samples; loadings plot (B) reflecting the influence of a particular parameter.

Variables with a correlation coefficient greater than 0.70 (absolute value) were determined in PC1. Those were TV (-0.872), LL (-0.773), LW (- 0.867), MDH (0.775), PGD (0.713), and SDH (-0.791). The summarisation of these traits in one component reflected the strong correlation between them reciprocally. On the other hand, PC2 was dominated only by GDH strong factor loading of -0.772.

The PCA correlation plots (Figure 1A) showed clustering of the genotypes into three main groups. The most influential in distinguishing the first group from other genotypes, (represented by strong vigorous *P. avium* genotypes, and other genotypes where *P. avium* is one of the progenitors [Colt and MaxMa 14] accompanied with one *P. mahaleb* genotype [TIKA]) were LL, LW, TV and ADH. Moving from the left side of the PC1 axis to the right, tree vigour and leaf size decreased in *P. cerasus* and P. *fruticosa.* The most important for the formation of the second group, which consisted of low vigorous *P. cerasus* genotypes ('Oblačinska' sour cherry clones), and genotypes derived from the crossing combination where *P. cerasus* was involved (Gisela 5 and Gisela 6) and one *P. fruticosa* (SV3) genotype, were IDH and MDH genotypes. The third group consisted of some *P. fruticosa* (SV) genotypes characterised by low vigour and small leaves, and separated based on the PGD zymogram pattern. The scores plot revealed one sample of *P. mahaleb* (BNS) to be an outlier due to the extremely strong vigour and some unique zymogram patterns (GDH and SDH) compared with the other genotypes.

Our findings support the results of the study previously conducted by Ognjanov et al. (2012), who have reported that the tree vigour and leaf shape characteristics are the most reliable parameters for the assessments of reduced growth in sour cherry. On the other hand, in this study, the most influential isoenzyme systems which led to the separation of low vigorous genotypes were IDH, MDH and PGD.

Important correlations were found among the studied traits and they are presented in Table 2. Our results showed a positive and significant correlation between TV and LL, as well as between TV and LW (r = 0.513 and r = 0.674). Therefore, these parameters may be used to predict each other. Contrary to this, Rakonjac et al. (2014), while studying wild cherry genotypes, found no significant correlations between those traits, which is probably due to the diverse genotypes studied.

Table 2. Correlations among variables significant at the 5% (*) level.

	LL[a]	LW	ADH	GDH	IDH	MDH	PGD	SDH
TV	0.513*	0.674*	0.619*	0.467*	-0.261	-0.641*	-0.465*	0.767*
LL		0.882*	0.342	-0.106	-0.102	-0.544*	-0.831*	0.329
LW			0.616*	-0.013	-0.082	-0.517*	-0.826*	0.452*
ADH				0.081	-0.115	-0.321	-0.276	0.524*
GDH					-0.325	-0.271	0.143	0.607*
IDH						0.467*	0.029	-0.575*
MDH							0.448*	-0.629*
PGD								-0.311

*Significant difference; [a] For explanation of character symbols, see 'Material and methods'.

We also observed a significant positive correlation between tree vigour and ADH, GDH and SDH banding patterns (r = 0.619, r = 0.467 and r = 0.767, respectively), whereas a significant, but negative correlation between MDH and PGD (r = -0.641 and r = -0.465, respectively) was recorded. On the other hand, LL and LW correlated strongly, but negatively, only with MDH and PGD, while LW showed a positive correlation with SDH. Finally, only MDH and PGD correlated (negatively) with all tree/leaf parameters. An established correlation between tree vigour and MDH is in accordance with the results of Werner and Moxley (1991) reported for peach.

Conclusion

Regarding all results obtained in this study, it can be concluded that PCA provided a simplified classification of the cherry rootstocks, and determined the groups of strong, medium and low vigour, while the scatter plot showed the geometrical distances among the groups. Both PCA and correlation analysis determined that MDH and PGD were correlated to TV, LL and LW, which suggests that those systems can be used for early selection of low vigour rootstocks. This conclusion is also supported by the isoenzyme analysis, which showed that MDH analysis could be applied in early rootstock selection, since MDH heterozygous genotype *ab* is specific for low vigour genotypes. PGD seems to be a good marker for selections of genotypes with small leaves that can be interesting for the selection of decorative forms. In order to confirm our results, further research should expand on new, even more dwarfing, precocious cherry rootstocks and/or test them in different varieties.

Acknowledgements

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POLIMORFIZAM DEHIDROGENAZA KAO ALAT ZA RANU SELEKCIJU SLABO BUJNIH PODLOGA ZA TREŠNJU I VIŠNJU

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R e z i m e

U radu je ispitivana korelacija između dehidrogenaznih izoenzimskih profila i bujnosti kod 20 genotipova roda *Prunus*, podroda *Cerasus*, kao i kod četiri komercijalne podloge: Colt, Gisela 5, Gisela 6 i MaxMa 14. Analiza glavnih komponenti je primenjena kako bi se ocenila potencijalna primena izoenzima za ranu selekciju slabo bujnih podloga za trešnju i višnju. Najveći uticaj na izdvajanje slabo bujnih genotipova imali su izoenzimski sistemi izocitrat dehidrogenaza, malat dehidrogenaza i fosfoglukonat dehidrogenaza. Značajna negativna korelacija utvrđena je između bujnosti i malat dehidrogenaze genotipa *ab*, kao i između bujnosti i fosfoglukonat dehidrogenaze, što ove sisteme čini potencijalnim markerima za ranu selekciju slabo bujnih podloga.

Ključne reči: preselekcija, bujnost, podloga, izoenzimi, korelacija, PCA.

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